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Neuropilin-2: Novel Biomarker and Therapeutic Target for Aggressive Prostate Cancer

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14. ABSTRACT

VEGF/NRP2 signaling is critical for the function of prostate cancer stem cells and a prime target for therapy. Surprisingly, however, we observed that that CSCs isolated from prostate tumors are resistant to anti-VEGF (bevacizumab) therapy. To understand this discrepancy, we generated prostate cancer cells that are resistant to bevacizumab and observed that resistant cells exhibit properties of CSCs compared to sensitive cells. Resistance is mediated by VEGF/NRP2 signaling, which is not inhibited by bevacizumab, and it involves the induction of P-Rex1, a Rac GEF, and consequent Rac1-mediated ERK activation. CSCs isolated from the $PTEN^{pc-/-}$ transgenic model of prostate cancer exhibit Rac1-dependent resistance to bevacizumab. Rac1 inhibition or P-Rex1 down-regulation increases the sensitivity of prostate tumors to bevacizumab. Thus, prostate tumors harbor cells with CSC properties that are resistant to inhibitors of VEGF/VEGFR signaling. Combining the use of available VEGF/VEGFR-targeted therapies with Rac1 inhibition should improve the efficacy of these therapies significantly.

15. SUBJECT TERMS
Prostate cancer, VEGF, Neuropilin, IGF-1 receptor, PTEN, stem cells, therapy

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1. INTRODUCTION: This award is based on the premise that prostate carcinoma (PCa) cells express receptors for VEGF and that these receptors contribute to tumor initiation (1). Our focus is on Neuropilin-2 (NRP2), a VEGF receptor that is not expressed in normal prostate but is expressed in PCa and correlates with Gleason grade (2). PTEN deletion induces NRP2 expression and NRP2 contributes to PCa formation (2). The role of VEGF/NRP2 signaling in prostate tumorigenesis can be explained by our discovery that NRP2 facilitates the expression of Bmi-1, a transcriptional repressor that has a critical role in the function of PCa stem cells (2). We demonstrated that NRP2 suppresses the IGF-1 receptor (IGF-1R) by a mechanism that involves transcriptional repression by Bmi-1 and, as a consequence, confers resistance to IGF-1R therapy of prostate carcinoma (2). This finding is significant because several IGF-1R inhibitors are in clinical trials (3) but the mechanisms to account for patient response to these inhibitors are largely unknown. Similarly, clinical trials of the VEGF Ab bevacizumab have been disappointing for reasons that are not entirely known (4). In the third year of this award, we investigated the problem of why VEGF-function blocking antibodies such as bevacizumab are not effective in treating prostate cancer, especially given our central hypothesis that VEGF/NRP2 signaling is essential for the function of PCa stem cells. These studies led to the discovery a novel mechanism of resistance to VEGF/VEGR receptor-targeted therapy that involves NRP2.

2. KEYWORDS: Prostate cancer, VEGF, Neuropilin, IGF-1 receptor, PTEN, stem cells, therapy

3. OVERALL PROJECT SUMMARY: During the tenure of this award, we made progress on the following tasks:

Task 1. Establish that VEGF/NRP2 signaling contributes to the function of tumor-initiating cells and the formation of prostate carcinoma induced by PTEN deletion. We completed Task 1. The breeding events outlined in the original proposal have been completed and we have analyzed the prostate tumors that were generated. Specifically, we found that conditional deletion of NRP2 in $PTEN^{pc-/-}$ transgenic mice delays the development of prostate intraepithelial neoplasia (PIN) and prostate adenocarcinoma significantly. These results provide a rigorous validation of our hypothesis that NRP2 has an essential role in the genesis of prostate cancer. These data also complete Tasks 1a and 1b in our SOW. Subsequently, we purified the stem-like cells [($Lin^-Sca\text{-}1^+CD49f^{high}$ cells), referred to as LSC cells] from the $NRP2^{loxP/loxP}/PTEN^{loxP/loxP}$/PB-Cre and $NRP2^{wt}/PTEN^{loxP/loxP}$/PB-Cre tumors by FACS. Our results revealed that conditional deletion of NRP2 caused a significant decrease in the frequency of LSC cells. Also, analysis of the Lin^- cells indicated that NRP2 expression is essentially ablated in this population obtained from the $NRP2^{loxP/loxP}/PTEN^{loxP/loxP}$/PB-Cre tumors compared to the control tumors and the ability of this population to form prostatospheres is dramatically reduced. Together, these data provide convincing and rigorous evidence that NRP2 is necessary for the genesis and function of prostate cancer stem cells. These results also complete Tasks 1c and 1d in our SOW.

We also addressed the critical issue of why FDA-approved drugs that target VEGF and VEGF receptors are relatively ineffective for the treatment of prostate cancer, especially given our finding that VEGF/NRP2 signaling is essential for the function of prostate CSCs. These data have been published (**Goel et al., Cell Reports, 2016**). We discovered that CSCs isolated from human prostate tumors are resistant to bevacizumab (a VEGF antibody) and sunitinib (a VEGF receptor tyrosine kinase inhibitor) [**see Figures 1 and 2 in** (**Goel et al., Cell Reports, 2016**)]. To understand the mechanistic basis for this resistance, we generated cell lines (PC3 and C4-2) that are resistant to bevacizumab and observed that resistant cells are enriched for stem cell properties compared to sensitive cells [**see Figure 2 in** (**Goel et al., Cell Reports, 2016**)]. This conclusion was substantiated by comparing the gene expression profiles of sensitive and resistant cells [**see Figure 2 in** (**Goel et al., Cell Reports, 2016**)]. Interestingly, this analysis also revealed that the expression of VEGF and NRP2 was elevated significantly in resistant cells compared to sensitive cells. This finding suggested that resistance to bevacizumab and sunitinib is actually mediated by VEGF/NRP2 signaling. Indeed, inhibiting the VEGF/NRP2 interaction using a specific peptide (c-furSEMA) caused resistant cells to be sensitive to bevacizumab [**see Figure 3 in** (**Goel et al., Cell Reports, 2016**)]. Subsequently, we investigated how VEGF/NRP2 signaling promotes resistance to bevacizumab and discovered that the GTPase Rac1 is activated by this signaling and contributes to resistance [**see Figure 3 in** (**Goel et al., Cell Reports, 2016**)]. Furthermore,

our experiments revealed that P-Rex1, a specific guanine-nucleotide exchange factor, is responsible for mediating resistance to bevacizumab [**see Figure 4 in (Goel et al., Cell Reports, 2016)**].

Task 2. Establish that NRP2 represses the IGF-1R and assess the consequences of this regulation on the function of prostate carcinoma cells. We hypothesized in the original proposal that VEGF/NRP2 signaling represses the IGF-1R and that the IGF-1R actually antagonizes the function of prostate CSCs because it promotes differentiation. In Years 1 and 2 of this award, we obtained and published data that support this hypothesis (**Goel et al, Cancer Discovery, 2012**). A critical finding was made in this study. Specifically, we observed that IGF-1R expression is markedly suppressed in prostate tumor cells that are resistant to bevacizumab and exhibit CSC properties **[see Figure 4 in (Goel et al, Cancer Discovery, 2012)**]. This finding substantiates our hypothesis that VEGF/NRP2 signaling represses the IGF-1R and that IGF-1R therapy by itself would not be effective at targeting prostate CSCs **[see Figure 4 in (Goel et al, Cancer Discovery, 2012)**]. These results complete Task 2 in our SOW.

Task 3. Evaluate the relationship between NRP2 and IGF-1R in PCa therapy. A central premise of Task 3 is that VEGF/NRP2 signaling is a prime target for therapy because it is essential for the function of PCa CSCs. As mentioned above, however, targeting VEGF is not effective because PCa CSCs are dependent on VEGF/NRP2 signaling and the available VEGF function blocking antibodies do not inhibit the binding of VEGF to NRP2. The data we reported for Task 1, however, indicate the possibility that inhibition of either Rac1 or P-Rex1 in resistant cells would render them sensitive to VEGF-function blocking antibodies. This important conclusion was verified using both xenografts of the resistant cell lines and the $PTEN^{pc-/-}$ model of prostate cancer [**see Figures 4 and 5 in (Goel et al., Cell Reports, 2016)**]. Given that the IGF-1R is highly expressed in cells that are sensitive to bevacizumab [**see Figure 2 in (Goel et al., Cell Reports, 2016)**], our results indicate that targeting of VEGF and the IGF-1R in combination with P-Rex1/Rac1 inhibition would be a very effective therapeutic strategy [**see Figures 4 and 5 in (Goel et al., Cell Reports, 2016)**]. These results complete Task 3 in our SOW.

4. KEY RESEARCH ACCOMPLISHMENTS:

- Demonstrated that prostate CSCs are resistant to drugs that target VEGF (bevacizumab) and VEGF receptor tyrosine kinases (sunitinib).
- Demonstrated that resistance to VEGF-targeted therapy is actually mediated by VEGF/NRP2 signaling.
- Obtained rigorous evidence that the ability of VEGF/NRP2 signaling to induce P-Rex1 expression and activate Rac1 is responsible for resistance to bevacizumab.
- Obtained rigorous and unbiased evidence that expression of the IGF-1R is suppressed in cells that are resistant to bevacizumab and elevated in cells that are sensitive.
- Demonstrated that inhibition of P-Rex1 or Rac1 renders resistant cells sensitive to bevacisumab, and that the combination of Rac1 and VEGF inhibition is highly effective at reducing prostate tumor initiation.

5. CONCLUSIONS:

We have completed all of the goals outlined in the SOW.

6. PUBLICATIONS, ABSTRACTS AND PRESENTATIONS:

Goel HL, Chang C, Pursell B, Leav I, Lyle SR, Xi HS, Hsieh CC, Adisetiyo H, Roy-Burman P, Coleman IM, Nelson PS, Vessella RL, Davis R, Plymate SR, Mercurio AM. VEGF/Neuropilin-2 regulation of Bmi-1 and repression of IGF-1R define a novel mechanism of aggressive prostate cancer. **Cancer Discovery**, 2012, 2:906-921.

Mercurio AM, Goel HL. VEGF targets the tumor cell. **Nature Reviews Cancer**, 2013, 13:871-882.

Parker MW, Linkugel AD, Goel HL, Wu T, Mercurio AM, Vander Kooi CW. Structural basis for VEGF-C binding to neuropilin-2 and sequestration by a soluble slice form. **Structure** 2015, 23:677-687.

Goel HL, Pursell B, Shultz LD, Greiner DL, Brekken RA, Vander Kooi CW, **Mercurio AM**. P-Rex1 promotes resistance to VEGF-targeted therapy in prostate cancer by mediating neuropilin signaling. **Cell Reports**, 2016, 14:2193-2208.

7. INVENTIONS, PATENTS AND LICENSES: A provisional patent application was filed by UMASS Medical School on February 18, 2016 for the combined use of Rac1 and VEGF inhibitors for the treatment of prostate cancer has been filed.

8. REPORTABLE OUTCOMES:

- Generation of a transgenic mouse model of prostate cancer in which NRP2 has been conditionally deleted.
- Development of a model for studying resistance to therapy in prostate cancer.
- Development of a novel therapeutic strategy for prostate cancer that overcomes resistance to existing therapies.

9. OTHER ACHIEVEMENTS:

- Development of prostate cancer cell lines that are either sensitive or resistant to therapy (bevacuzimab; sunitinib and pazopanib). These cell lines provide a useful system for studying resistance to therapy and the importance of VEGF/NRP2 signaling in the acquisition of stem cell properties.

10. REFERENCES

1. Goel HL & Mercurio AM (2013) VEGF targets the tumour cell. *Nat Rev Cancer* 13(12):871-882.
2. Goel HL*, et al.* (2012) VEGF/neuropilin-2 regulation of Bmi-1 and consequent repression of IGF-IR define a novel mechanism of aggressive prostate cancer. *Cancer Discov* 2(10):906-921.
3. Gualberto A & Pollak M (2009) Emerging role of insulin-like growth factor receptor inhibitors in oncology: early clinical trial results and future directions. *Oncogene* 28(34):3009-3021.
4. Ning YM*, et al.* (2010) Phase II trial of bevacizumab, thalidomide, docetaxel, and prednisone in patients with metastatic castration-resistant prostate cancer. *J Clin Oncol* 28(12):2070-2076.

11. SUPPORTING DATA

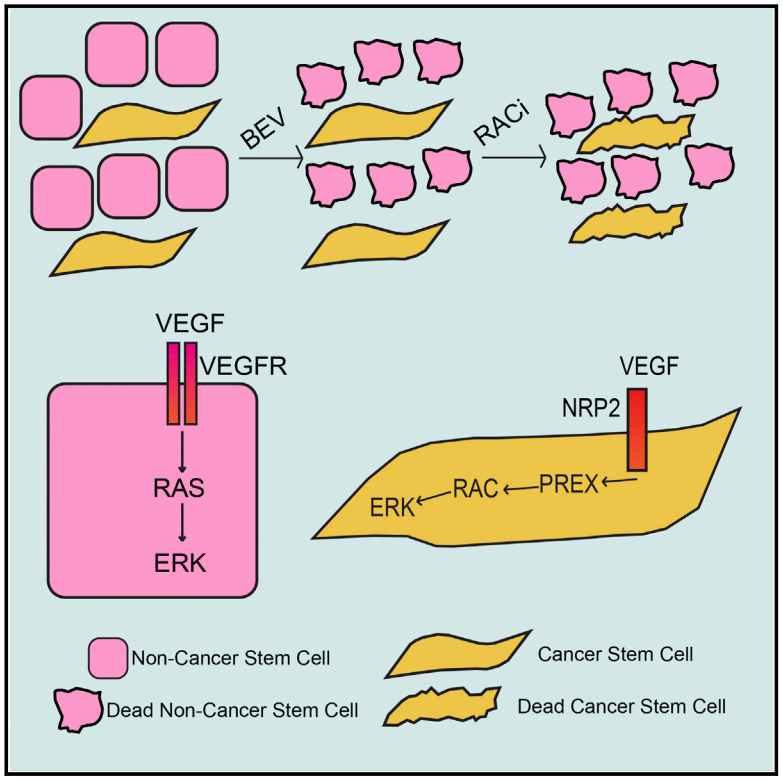
The publications mentioned above have been appended to this final report.

Article

Cell Reports

P-Rex1 Promotes Resistance to VEGF/VEGFR-Targeted Therapy in Prostate Cancer

Graphical Abstract



Highlights

- Prostate tumor cells resistant to bevacizumab have stem cell properties
- Resistance is mediated by VEGF/neuropilin signaling
- Neuropilin signaling induces P-Rex1 and activates Rac1, which sustains resistance
- Inhibition of P-Rex1 or Rac1 renders tumor cells sensitive to bevacizumab

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In Brief

Understanding mechanisms of resistance to targeted therapy is critical. Goel et al. demonstrate that resistance to bevacizumab occurs by neuropilin signaling and activation of P-Rex1/Rac1. Inhibition of these molecules increases the sensitivity of prostate tumors to bevacizumab.

Goel et al., 2016, Cell Reports *14*, 1–16
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CellPress

Cell Reports
Article

P-Rex1 Promotes Resistance to VEGF/VEGFR-Targeted Therapy in Prostate Cancer

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http://dx.doi.org/10.1016/j.celrep.2016.02.016

SUMMARY

Autocrine VEGF signaling is critical for sustaining prostate and other cancer stem cells (CSCs), and it is a potential therapeutic target, but we observed that CSCs isolated from prostate tumors are resistant to anti-VEGF (bevacizumab) and anti-VEGFR (sunitinib) therapy. Intriguingly, resistance is mediated by VEGF/neuropilin signaling, which is not inhibited by bevacizumab and sunitinib, and it involves the induction of P-Rex1, a Rac GEF, and consequent Rac1-mediated ERK activation. This induction of P-Rex1 is dependent on Myc. CSCs isolated from the PTEN$^{pc-/-}$ transgenic model of prostate cancer exhibit Rac1-dependent resistance to bevacizumab. Rac1 inhibition or P-Rex1 downregulation increases the sensitivity of prostate tumors to bevacizumab. These data reveal that prostate tumors harbor cells with stem cell properties that are resistant to inhibitors of VEGF/VEGFR signaling. Combining the use of available VEGF/VEGFR-targeted therapies with P-Rex1 or Rac1 inhibition should improve the efficacy of these therapies significantly.

INTRODUCTION

We are interested in the contribution of vascular endothelial growth factor (VEGF) and its receptors to prostate cancer and the potential for VEGF-targeted therapies in the treatment of this common cancer. Expression of VEGF is elevated in aggressive prostate cancer (Tomić et al., 2012), and a recent meta-analysis identified high VEGF expression as a prognostic factor for poor overall survival in men with prostate cancer (Wang et al., 2012). These and other data indicate that VEGF and VEGF receptors are feasible therapeutic targets. In fact, bevacizumab, a humanized VEGF antibody that blocks VEGF interactions with its tyrosine kinase receptors (VEGFRs) (Merino et al., 2011), and sunitinib, an inhibitor of VEGFRs and other receptors (Michaelson et al., 2014), have been used in clinical trials of prostate cancer patients (Merino et al., 2011). The prevailing assumption in these studies has been that these drugs target tumor angiogenesis (Merino et al., 2011; Goel and Mercurio, 2013). These trials did not yield a significant survival advantage, which has discouraged the use of these inhibitors for this disease. For example, the results from bevacizumab monotherapy were very disappointing, with no response noted based on RECIST (Response Evaluation Criteria in Solid Tumors) criteria, although 27% of patients exhibited a decline in prostate-specific antigen (Reese et al., 2001). A recent study of 873 patients with aggressive prostate cancer found that the addition of sunitinib to prednisone did not improve overall survival compared with placebo (Michaelson et al., 2014).

The reasons for the poor response to VEGF-targeted therapy in prostate cancer are not well understood but need to be considered in the context of the complexity of VEGF signaling in cancer. In addition to its contribution to endothelial biology and angiogenesis, VEGF signaling in tumor cells has emerged as an important factor in tumor initiation and progression (Goel and Mercurio, 2013; Chatterjee et al., 2013). More specifically, compelling evidence now exists that autocrine VEGF signaling is necessary for the function of cancer stem cells (CSCs) in prostate and other cancers (Goel and Mercurio, 2013; Goel et al., 2012). Given that CSCs have been implicated in resistance to therapy, tumor recurrence, and metastasis (Craft et al., 1999; Chen et al., 2013), this role for VEGF signaling is significant and it appears to be independent of its function as a mediator of tumor angiogenesis. The hypothesis can be formulated from this information that the poor response of prostate tumors, especially aggressive tumors, to anti-VEGF (bevacizumab) and anti-VEGR therapy is that these therapies do not target CSCs effectively despite the fact that they are dependent on VEGF signaling. In this study, we pursued this hypothesis and sought to investigate the mechanisms involved.

RESULTS

Cells with Stem-like Properties Are Resistant to Anti-VEGF/VEGFR Therapies

To assess the sensitivity of prostate CSCs to anti-VEGF therapy, we isolated a CD44^{+}CD24^{-} population from two freshly harvested

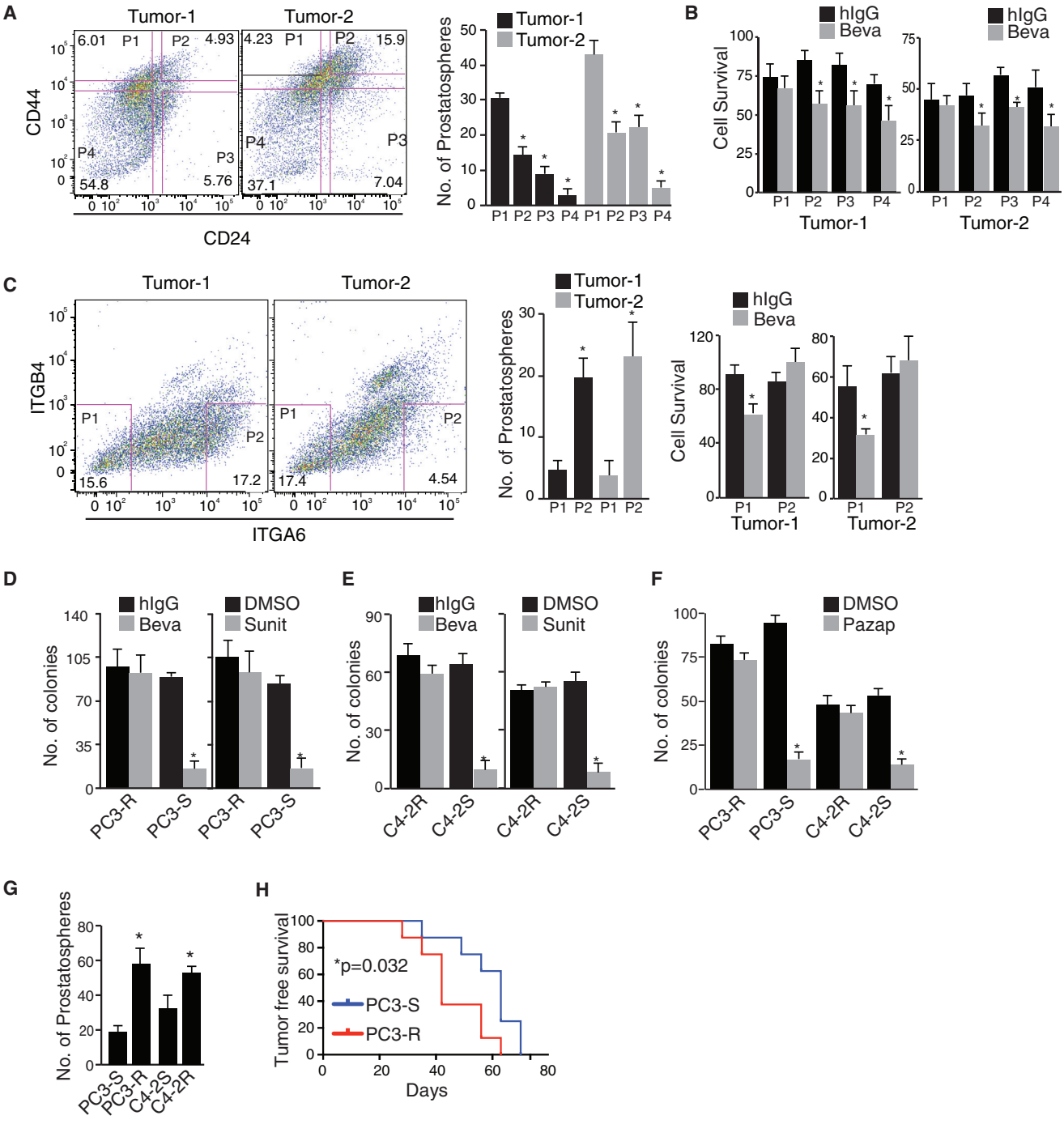

Figure 1. Characterization of Prostate Cancer Cells Resistant to VEGF-Targeted Therapy

(A and B) Cells from two human prostate tumors were sorted using CD44 and CD24 antibodies (A). The four subpopulations isolated based on expression of CD44 and CD24 were analyzed for their sensitivity to bevacizumab (B) and ability to form prostatospheres (A).

(C) Cells from two human freshly harvested prostate tumors were sorted using ITGA6 and ITGB4 antibodies. The four subpopulations isolated based on expression of ITGA6 and ITGB4 were analyzed for their ability to form prostatospheres and sensitivity to bevacizumab. For (B) and (C), the percentage of live cells in three different areas was determined and mean is plotted as cell survival.

(D and E) PC3 and C4-2 sensitive and resistant cells (1,000 cells per 60-mm plate) were cultured in the presence of bevacizumab (1 mg/ml), sunitinib (20 μM), or their respective controls for 10 days, colonies were stained with crystal violet, and colonies with more than 50 cells were counted.

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human prostate tumors. This population is enriched for progenitor/stem cells (Hurt et al., 2008). Indeed, the $CD44^{+}CD24^{-}$ (P1) sub-population isolated from these tumors formed significantly more prostatospheres than the other sub-populations (Figure 1A), and it is the only subpopulation that exhibited resistance to bevacizumab treatment (Figure 1B). We also sorted these prostate tumors based on expression of CD49f (α6 integrin), another stem cell marker (Colombel et al., 2012), and observed that the high-CD49f population formed significantly more prostatospheres and exhibited resistance to bevacizumab treatment compared to the low-CD49f population (Figure 1C).

To understand the mechanism behind the resistance of CSCs to bevacizumab, we exposed prostate cancer cell lines (PC3 and C4-2) to increasing concentrations of bevacizumab until this inhibitor no longer affected their survival ($\sim$6 months). To circumvent VEGF-independent or transactivation of VEGFRs, we subsequently exposed these cells to increasing concentrations of sunitinib, an inhibitor of VEGRs and other VEGFRs (Michaelson et al., 2014), along with bevacizumab. However, sunitinib did not have a significant effect on bevacizumab-resistant cells (data not shown). The resistant cell lines generated are referred to as PC3-R and C4-2R. As controls, we also exposed these cell lines to control immunoglobulin G and DMSO and refer to these as sensitive cell lines (PC3-S and C4-2S) (Figures 1D and 1E).

Neither bevacizumab nor sunitinib inhibited the ability of the resistant cell lines to form colonies or survive, in contrast to the sensitive cell lines (Figures 1D, 1E, and S1A–S1D). Interestingly, PC3-R and C4-2R cells are also resistant to pazopanib, another VEGFR inhibitor (Figures 1F, S1E, and S1F), confirming the pathway specificity of the observed resistance. The resistant cell lines we generated are enriched for stem cell properties based on the fact that they were able to form prostatospheres and initiate tumors in NSG mice to a significantly greater extent than the sensitive cells (Figures 1G and 1H).

Neuropilin-Mediated Rac1 Activation Promotes Resistance to VEGF-Targeted Therapy

We compared the expression of key stem cell genes between the sensitive and resistant cell lines to substantiate our hypothesis that resistant cells exhibit stem cell properties. Indeed, the resistant cell lines are enriched in the expression of genes associated with CSCs (Nanog, Sox2, BMI1, and ALDH1) compared to the sensitive cell lines (Figure 2A). Interestingly, VEGF expression is markedly elevated in the resistant cell lines despite the fact that these cells were selected based on their resistance to bevacizumab. In contrast, no significant difference was observed in VEGFR2 expression between sensitive and resistant cells, and these cells lack expression of VEGFR1 (Figure 2A). Downregulation of VEGF expression in resistant cells reduced their ability to form colonies, suggesting that VEGF signaling contributes to bevacizumab and sunitinib resistance in a VEGFR2-independent manner (Figures S2A and S2B). The nature of this signaling was indicated by the observation that neuropilin (NRP) expression, especially NRP2, is dramatically elevated in resistant cell lines (Figures 2A and S2C). These expression data raised the possibility that VEGF/NRP signaling is responsible for resistance to bevacizumab and sunitinib, especially given the fact that bevacizumab blocks the interaction of VEGF with VEGFRs (VEGFR1–3), but not with NRPs (Geretti et al., 2010). The observation that IGF-1R expression is reduced dramatically in resistant cells (Figure 2A) is consistent with our previous finding that VEGF/NRP2 signaling represses IGF-1R transcription (Goel and Mercurio, 2013; Goel et al., 2012).

The contribution of NRPs to resistance was investigated using c-furSEMA, an inhibitory peptide, which blocks interactions of VEGF with NRPs (Parker et al., 2010). This peptide inhibited formation of prostatospheres in resistant cell lines and showed no effect in sensitive cells (Figure 2B). Importantly, treatment with c-furSEMA or an inhibitory NRP2 antibody decreased colony formation, highlighting a critical role for NRPs in the survival of resistant cells (Figure S2D). We also observed that inhibition of VEGF-NRP binding using c-furSEMA increased the sensitivity of resistant cells to bevacizumab, substantiating the critical function of NRPs in resistance to this VEGF inhibitor (Figure 2C). Furthermore, downregulation of either NRP2 or NRP1 significantly reduced colony formation and increased sensitivity to bevacizumab (Figure S2E). We focused on NRP2 for subsequent experiments based on our previous work (Goel and Mercurio, 2013; Goel et al., 2012) and the observation that NRP2 downregulation had a more potent inhibitory effect on colony formation than NRP1 (Figure S2E). Ectopic expression of NRP2 in sensitive cells induces resistance to bevacizumab in the presence of VEGF, directly implicating NRP2 in resistance to bevacizumab (Figure S2F).

Based on our finding that VEGF/NRP signaling promotes resistance to VEGF/VEGFR-targeted therapy, we investigated the details of this signaling mechanism. Initially, we compared activation of AKT and extracellular signal-regulated kinase (ERK) in sensitive and resistant cell lines, in the absence or presence of exogenous VEGF. Sensitive cells exhibited increased ERK activation in response to VEGF, which was inhibited by bevacizumab (Figure 2D). In contrast, resistant cells displayed relatively high ERK activation even in the absence of exogenous VEGF (Figure 2E), presumably the consequence of autocrine VEGF secretion in these cells. Interestingly, bevacizumab was unable to inhibit ERK activation in resistant cells (Figures 2F and 2G), suggesting that VEGF can induce ERK activation in these cells independently of VEGFR. No differences in AKT activation were observed between sensitive and resistant cells (Figures 2F and 2G). Since bevacizumab does not block the interaction of VEGF with NRP (Geretti et al., 2010), we expressed NRP2 in sensitive cells and observed that it induced ERK activation in the presence of bevacizumab (Figures 2H and 2I). This result implicates VEGF/NRP2 signaling in ERK activation. Interestingly, RAS does not appear to be involved in this mode of

(F) PC3- and C4-2-resistant and sensitive cell lines were analyzed for colony formation in the presence or absence of 10 μM pazopanib.
(G) Resistant and sensitive PC3 and C4-2 populations were compared for their ability to form prostatospheres.
(H) Resistant and sensitive PC3 populations were implanted into NSG mice, and tumor onset was plotted.
Error bars represent mean $\pm$ SD. Beva, bevacizumab; hIgG, control immunoglobulin G. See also Figure S1.

A

	C4-2S	C4-2R	PC3-S	PC3-R
STEMNESS				
OCT 4	1 (0.03)	0.98 (0.02)	1 (0.07)	3.3 (0.06)
Bmi-1	1 (0.03)	2.9 (0.042)	1 (0.05)	7.6 (0.43)
GLI1	1 (0.07)	4.2 (0.28)	1 (0.09)	8.5 (0.57)
KLF4	1 (0.21)	1.39 (0.09)	1 (0.08)	0.91 (0.18)
NANOG	1 (0.042)	3.1 (0.04)	1 (0.03)	15 (1.99)
SOX2	1 (0.17)	4.8 (0.139)	1 (0.42)	5.6 (0.84)
ALDH1	1 (0.46)	2.2 (0.68)	1 (0.19)	1.9 (0.48)
ALDH2	1 (0.8)	1.6 (0.6)	1 (0.23)	1.15 (0.63)
ALDH3	1 (0.02)	0.9 (0.45)	1 (0.02)	1.4 (0.8)
Snail1	1 (0.1)	1.6 (0.5)	1 (0.09)	2.6 (0.12)
VEGF	1 (0.05)	8.7 (0.2)	1 (0.03)	6.3 (0.7)
GROWTH FACTOR RECEPTOR				
c-Met	1 (0.13)	1.04 (0.36)	1 (0.037)	1.17 (0.42)
EGFR	1 (0.055)	0.5 (0.02)	1 (0.01)	3.6 (1.89)
IGF1R	1 (0.035)	0.12 (0.05)	1 (0.049)	0.18 (0.07)
IR	1 (0.04)	1.23 (0.52)	1 (0.15)	2.2 (1.11)
VEGFR1	0	0	0	0
VEGFR2	1.18 (0.68)	0.68 (0.12)	1 (0.23)	1.5 (0.58)
NPR1	1 (0.02)	6.3 (0.06)	1 (0.01)	6.11 (0.44)
NPR2	1 (0.042)	17.7 (1.56)	1 (0.27)	15.08 (1.99)

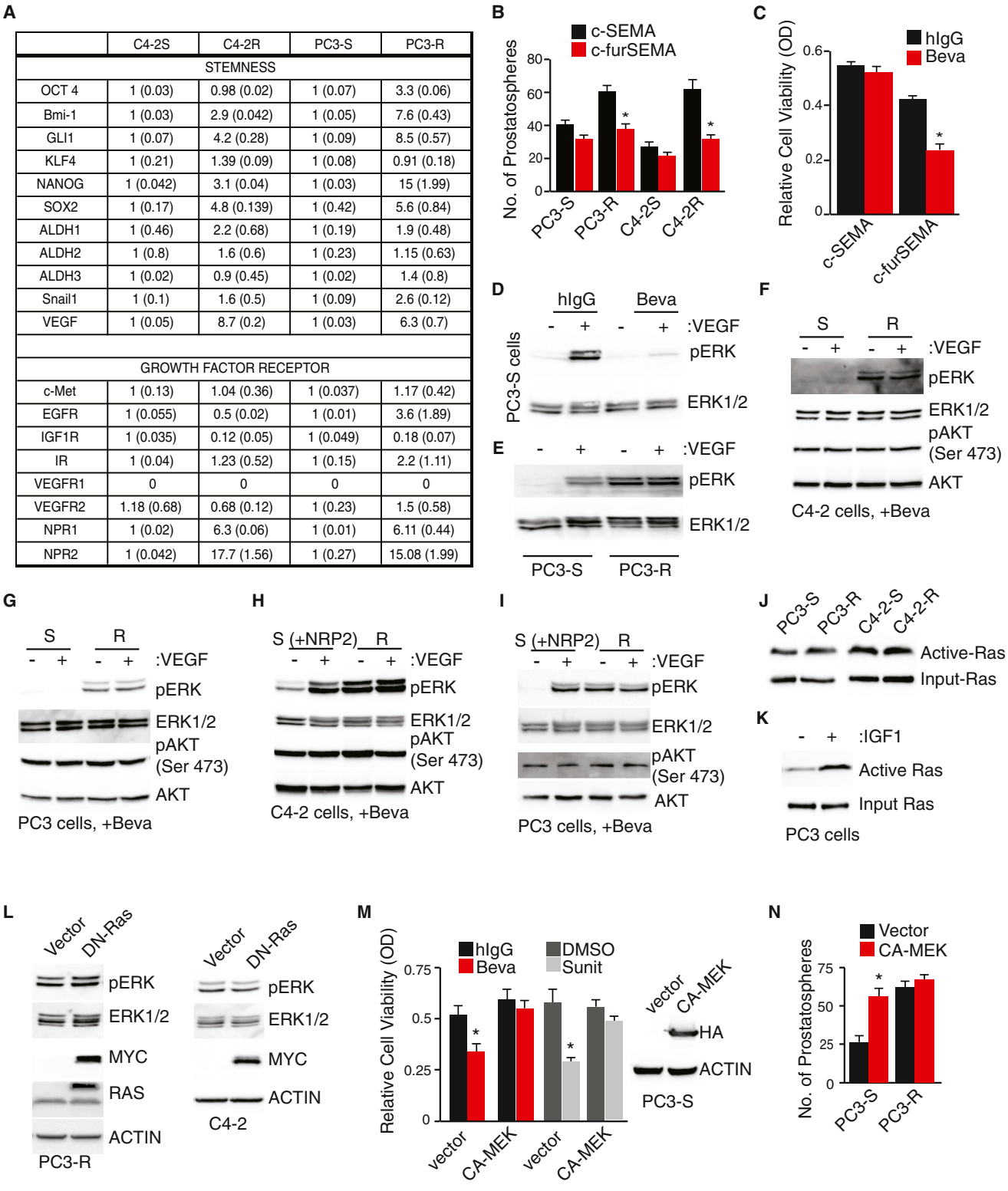

Figure 2. VEGF/NRP-Mediated Activation of ERK Promotes Resistance to Therapy

(A) Expression of CSC-related genes and growth factor receptors was quantified by qPCR in resistant and sensitive populations of PC3 and C42 cells. Tables show fold change in mRNA expression upon normalization with sensitive populations, which was set as 1.

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ERK activation based on the findings that no differences in the levels of active RAS were detected between sensitive and resistant cells (Figures 2J and 2K) and that expression of a dominant-negative RAS (DN-RAS) did not alter ERK activation in resistant cells (Figure 2L). ERK activation contributes to resistance based on the finding that expression of constitutively active MEK in sensitive cells increased their resistance to bevacizumab and sunitinib-mediated inhibition of viability and prostatosphere formation (Figures 2M and 2N).

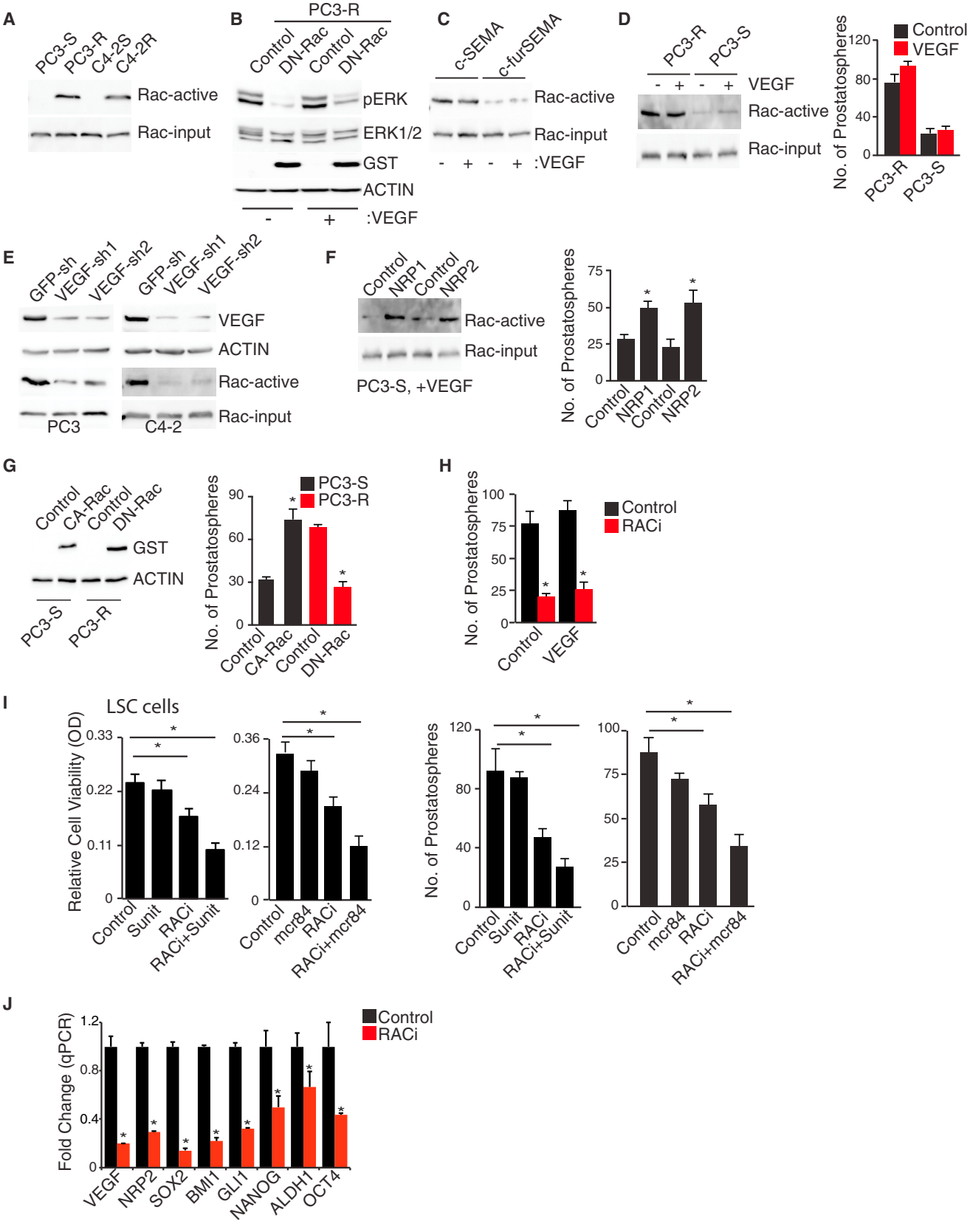
Subsequently, we focused on Rac1 as a mediator of Ras-independent ERK activation based on the reports that Rac1 is a major effector of NRP/plexin signaling (Liu and Strittmatter, 2001; Riccomagno et al., 2012) and plays a central role in vascular development in response to VEGF (Tan et al., 2008). Also, activation of Rac1 is associated with aggressive prostate cancer (Kobayashi et al., 2010), and $Rac1^{-/-}$ mice exhibit impaired ERK activation and regression of hematopoietic stem cells (Gu et al., 2003). Indeed, we found that resistant cell lines exhibit robust Rac1 activation compared to sensitive cells (Figure 3A). Rac1 mediates ERK activation in resistant cells based on the use of a dominant-negative Rac construct (Figure 3B). The activity of Rac1 in resistant cells is dependent upon NRP signaling because c-furSEMA reduced Rac1 activity significantly (Figure 3C). In contrast, addition of recombinant VEGF did not increase Rac1 activity or the ability of these cells to make prostatospheres (Figure 3D), most likely because resistant cells express high levels of autocrine VEGF (Figure 2A). This possibility was confirmed by depleting VEGF expression in these cells and observing a marked reduction in Rac1 activity (Figure 3E).

Sensitive cells may not respond to VEGF and activate Rac1 because they lack significant NRP expression. To test this possibility, we expressed either NRP1 or NRP2 in these cells and observed an increase in Rac1 activity and prostatosphere formation (Figure 3F). Also, expression of a constitutively active Rac1 in sensitive cells increased prostatosphere formation and expression of a dominant-negative Rac1 in resistant cells decreased their formation (Figure 3G). These results were confirmed using a Rac1 inhibitor (EHT1864) in resistant cells, which reduced the number of prostatospheres (Figure 3H). Although there is some indication that the ability of EHT1864 to inhibit Rac1 may be indirect (data not shown), we conclude from the use of dominant-negative and constitutively active Rac1 constructs, as well as EHT1864, that Rac1 is the primary mediator of VEGF/NRP-mediated prostatosphere formation.

To validate the role of Rac1 in tumor initiation, we utilized the $PTEN^{pc-/-}$ transgenic mouse model of prostate cancer (Mulholland et al., 2009). Tumors that form in this model harbor a small population of tumor initiating cells defined as $Lin^{-}Sca^{+}CD49f^{high}$ (referred to as $Lin^{-}Sca^{+}CD49f^{high}$ [LSC] cells) (Mulholland et al., 2009). We purified these LSC cells from 10-week-old $PTEN^{pc-/-}$ mice and observed increased expression of VEGF and NRP2 in this population compared to non-LSC cells (Figure S2G). We tested the hypothesis that Rac inhibition increases sensitivity to mcr84, which recognizes both mouse and human VEGF (Sullivan et al., 2010), and sunitinib. This antibody (mcr84) does not inhibit the interaction of VEGF with NRPs (Figure S2H). Consistent with our hypothesis, we observed that the Rac1 inhibitor increased the sensitivity of LSC cells to these drugs (Figure 3I). Inhibition of Rac1 also reduced the expression of VEGF, NRP2 and other stemness-related genes (Figure 3J).

The data in Figure 3I suggest that the response to VEGF-targeted therapy (bevacizumab or mcr84) would be improved significantly if Rac1 expression or activation were inhibited. To test this possibility initially, we treated control and Rac1-depleted PC3-R xenografts with bevacizumab or vehicle. Bevacizumab treatment alone had no significant effect on tumor growth, validating our in vitro finding that resistant cell lines can tolerate bevacizumab treatment. Although Rac1 inhibition reduced tumor volume, the combination of bevacizumab and Rac1 depletion resulted in a significantly better decrease in tumor volume (Figure 4A). Moreover, the residual tumors harvested from mice that received the combined treatment contained mostly apoptotic cells, in contrast to either bevacizumab treatment or Rac1 inhibition alone (Figure 4B). This unexpected observation suggests that resistant cells acquire sensitivity to bevacizumab as a result of Rac1 inhibition. Presumably, Rac1 inhibition alone reduces tumor growth but does not induce the massive apoptosis seen with combined treatment. To pursue this hypothesis further, $PTEN^{pc-/-}$ transgenic mice were treated with the Rac1 inhibitor (EHT1864), mcr84, or both at the start of puberty (6 weeks). Indeed, Rac1 inhibition reduced the number of LSC cells significantly but the combined treatment abolished the LSC population. We also compared the impact of mono- and

(B and C) Resistant and sensitive populations were analyzed for prostatosphere formation (B) or sensitivity to bevacizumab (1 mg/ml) (C) in the presence of either a NRP inhibitory peptide (c-furSEMA) or control peptide (c-SEMA).
(D) PC3-S cells were serum-deprived overnight and stimulated with VEGF (50 ng/ml) for 30 min in the presence or absence of bevacizumab (5 mg/ml). The activation of ERK was analyzed by immunoblotting using a phospho-specific antibody.
(E) PC3-sensitive or resistant cells were serum-deprived overnight and stimulated with VEGF (50 ng/ml) for 30 min. The activation of ERK was analyzed by immunoblotting using a phospho-specific antibody.
(F and G) Sensitive and resistant PC3 and C4-2 cell lines were serum-deprived overnight and stimulated with VEGF (50 ng/ml) for 30 min in the presence of bevacizumab (5 mg/ml). The activation of ERK and AKT was analyzed by immunoblotting using phospho-specific antibodies.
(H and I) NRP2 was expressed in sensitive populations of PC3 and C4-2 cells. These cells and resistant PC3 and C4-2 cells serum-deprived overnight and stimulated with VEGF (50 ng/ml) for 30 min in the presence of bevacizumab (5 mg/ml). The activation of ERK and AKT was analyzed by immunoblotting.
(J) Ras activation was analyzed in sensitive and resistant PC3 and C4-2 cell using the Raf1 binding assay.
(K) PC3-S cells were stimulated with IGF-1 (100 ng/ml) for 20 min and Ras activation was analyzed.
(L) Resistant PC3 and C4-2 cells were transfected with a Myc-tagged dominant-negative (DN) Ras construct, and ERK activation was analyzed by immunoblotting.
(M and N) Sensitive PC3 cells were transfected with a hemagglutinin (HA)-tagged, constitutively active (CA) MEK construct, and sensitivity to bevacizumab (M) and the effect on prostatosphere formation (N) were analyzed.
Error bars represent mean ± SD. Beva, bevacizumab; hIgG, control immunoglobulin G. See also Figure S2.



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combined therapy on PTEN$^{pc-/-}$ tumors by calculating the weights of the isolated genitourinary (GU) tracts and prostate lobes. Combined treatment (EHT1864 + mcr84) resulted in a significant decrease in the weight of the isolated GU tracts and prostate lobes compared to either EHT1864 or mcr84 alone (Figures 4C and 4D). Pathological examination revealed that tumors progressed to well-differentiated adenocarcinomas in mice that received either control or single-agent treatment. Interestingly, however, prostatic intraepithelial neoplasia (PIN) lesions were observed in the prostate glands of mice that received combined treatment (RACi + mcr84), suggesting a delay in tumor progression as a result of the reduced number of LSC cells (Figure 4E). Moreover, a mass of cells in the lumen of the gland was evident in mice that received the combined treatment. Further analysis using the TUNEL assay demonstrated that this mass of cells is apoptotic, indicating that combined treatment can induce apoptotic cell death within PIN lesions (Figures 4F and S3). We also stained these tumor groups with CD31 and observed no significant difference in staining among the groups, indicating that the observed impact of these treatments is not caused by an effect of these compounds on angiogenesis (Figure S4A).

Identification of P-Rex1 as the Mediator of Resistance to VEGF-Targeted Therapy

To understand how VEGF/NRP signaling activates Rac1 and promotes resistance to VEGF/VEGFR-targeted therapy, we compared the expression of potential guanine-nucleotide exchange factors (GEFs) known to be involved in Rac1 activity in sensitive and resistant cells (Figure 5A). This screening revealed elevated expression of P-Rex1 and, to as lesser extent, TIAM1 in resistant cells (Figure 5A). The importance of P-Rex1 in Rac1 activation is indicated by our finding that expression of exogenous P-Rex1 in VEGF-depleted resistant cells or in sensitive cells restored Rac1 activation (Figures 5B and S4B). In contrast, downregulation of TIAM1 expression in resistant cells had no effect on Rac1 activation (Figure 5C), suggesting that endogenous P-Rex1 is sufficient to maintain Rac1 activation even in the absence of TIAM1. For this reason, we focused subsequent experiments on P-Rex1. P-Rex1 expression is dependent upon VEGF/NRP signaling because downregulation of VEGF significantly reduced P-Rex1 expression in resistant cells (Figure 5D) and expression of either NRP1 or NRP2 in sensitive cells increased P-Rex1 expression (Figure 5E). Moreover, depletion of P-Rex1 expression in resistant cells diminished Rac1 activity and prostatosphere formation (Figure 5F). The importance of P-Rex1 in promoting resistance is indicated by the finding that downregulation of P-Rex1 in resistant cells increased their sensitivity to bevacizumab and sunitinib (Figure 5G).

Our P-Rex1 experimental results were validated by analyzing the gene expression profiles of epithelial cells micro-dissected from benign prostates and tumor cells from Pten-null prostate carcinomas (Garcia et al., 2014). P-Rex1 expression is significantly elevated in cancer cells compared to benign epithelium ($p = 0.04$) (Figure 5H). We also compared the expression levels of Rac GEFs in LSC and non-LSC cells isolated from PTEN$^{pc-/-}$ prostate tumors. Among all of the GEFs analyzed, only P-Rex1 expression is increased significantly in LSC compared to non-LSC cells (Figure 5I). P-Rex1 expression is higher in prostate adenocarcinoma compared to non-cancerous tissues (Qin et al., 2009). More specifically, we observed that P-Rex1 expression correlates with tumor grade (Figure 5J), similar to NRP2 expression (Goel et al., 2012). In fact, a positive correlation between P-Rex1 and NRP2 expression was detected in a cohort of prostate tumors (Figure 5J).

To demonstrate that VEGF-induced tumor initiation is dependent upon Rac1 activation, we engineered PC3 cells to express GFP under control of the VEGF promoter. We sorted these cells and generated two distinct populations designated VEGF^{high} and VEGF^{low} (Figure 6A). VEGF^{high} cells form more colonies in soft agar and initiate tumors more rapidly than VEGF^{low} cells (Figures 6B and 6C). Similar to the resistant cell lines described above, VEGF^{high} cells express high levels of genes associated with CSCs, NRPs, and P-Rex1 (Figure 6D). Also, the VEGF^{high} cells are more resistant to bevacizumab and sunitinib compared to the VEGF^{low} cells (Figure 6E). VEGF induces ERK activation, which is inhibited by bevacizumab in VEGF^{low} cells (Figure 6F). In contrast, VEGF^{high} cells exhibit high basal ERK activation and this activation is resistant to bevacizumab (Figure 6F). VEGF^{high} cells also exhibited increased Rac1 activity compared

Figure 3. Rac1 Mediates Stem Cell Properties and Resistance to VEGF-Targeted Therapy

(A) Rac1 activation was compared in resistant and sensitive PC3 and C4-2 cells.

(B) Resistant PC3 cells were transfected with a glutathione S-transferase (GST)-tagged dominant-negative (DN) Rac1 construct and stimulated with VEGF, and activation of ERK was analyzed by immunoblotting. GST expression indicates the level of DN-Rac expression.

(C) Rac1 activation was measured in resistant PC3 cells in response to VEGF treatment in the presence of either a NRP inhibitory peptide (c-furSEMA) or control peptide (c-SEMA).

(D) Resistant and sensitive PC3 cells were stimulated with VEGF and the effect on Rac1 activation and prostatosphere formation was measured.

(E) VEGF expression was diminished in resistant PC3 and C4-2 cells using two different shRNAs and the effect on Rac1 activation was determined.

(F) Either NRP1 or NRP2 was expressed in sensitive PC3 cells. These cells were stimulated with VEGF (50 ng/ml) for 30 min and the effect on Rac1 activation and prostatosphere formation was measured.

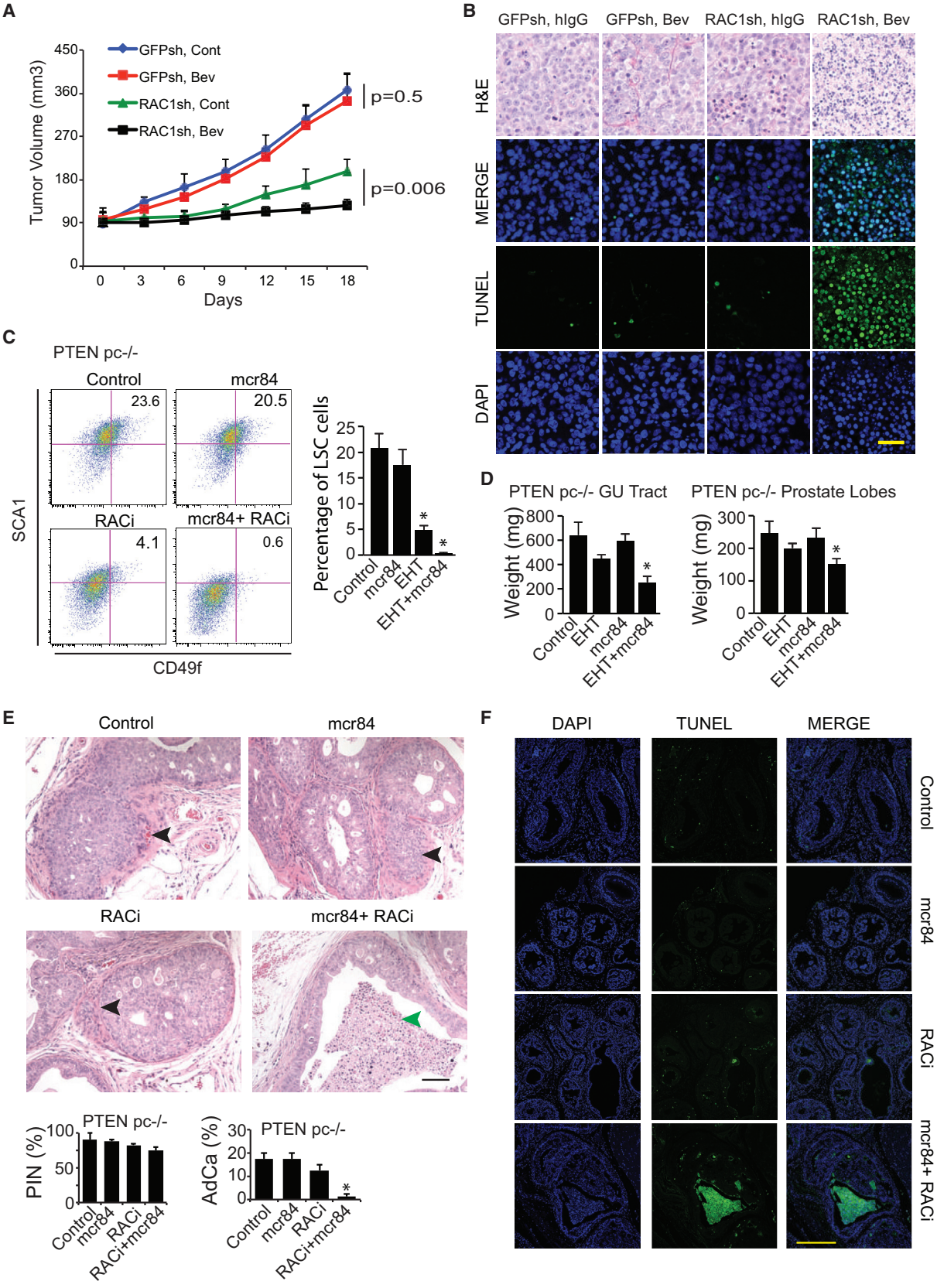
(G) Resistant and sensitive PC3 cells were transfected with a GST-tagged, dominant-negative Rac construct (DN-Rac) or a constitutively active Rac construct (CA-Rac), and their effect on prostatosphere formation was measured.

(H) PC3-R cells were stimulated with VEGF in the presence or absence of a Rac1 inhibitor (EHT1864; 20 μM) and the effect on prostatosphere formation was measured.

(I) Freshly sorted LSC cells from PTEN$^{pc-/-}$ mice were used to measure the effect of EHT1864, mcr84, or sunitinib on cell proliferation and prostatosphere formation.

(J) Freshly sorted LSC cells from PTEN$^{pc-/-}$ mice were treated with EHT1864 (20 μM), and expression of genes associated with stem cells and VEGF signaling was quantified by qPCR.

Error bars represent mean ± SD.



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to the VEGF^{low} cells (Figure 6G). Also, downregulation of NRP2 in VEGF^{high} cells reduced Rac1 activation (Figure 6H). Importantly, inhibition of Rac1 in VEGF^{high} cells reduced their ability to form prostatospheres in vitro and tumors in vivo (Figures 6I and 6J). Also, P-Rex1 downregulation reduced tumor onset in vivo (Figure 6K), confirming the crucial role of P-Rex1 in VEGF/NRP/Rac1 signaling. Taken together, these data substantiate the ability of VEGF/NRP2/P-Rex1 signaling to activate Rac1 and the importance of this pathway in tumor formation.

To identify the mechanism of P-Rex1 regulation, we focused on its transcriptional regulation, because we observed increased activity of a luciferase reporter construct containing the P-Rex1 promoter in resistant cells compared to sensitive cells (Figure 7A). We used the UCSC genome browser to search for putative transcription factor binding sites on the P-Rex1 promoter and identified Myc as a possible candidate. A role for Myc is supported by the increased expression of Myc in resistant compared to sensitive cell lines, as well as enrichment of Myc-positive cells in PTEN$^{pc-/-}$ tumors upon treatment with mcr84 (Figures 7B and 7C). Moreover, Myc downregulation reduced Rac1 activation and P-Rex1 expression in resistant cells (Figures 7D, 7E, S5A, and S5B). More definitively, we detected direct binding of Myc on the P-Rex1 promoter by ChIP (Figure 7F), and mutation of a putative myc-binding site (CACTTG, −246) significantly reduced the activity of a luciferase promoter construct (Figure S5C). We also found a significant correlation in P-Rex1 and Myc expression in human prostate cancer specimens by immunohistochemistry (Figures 7G and S5D). These results infer that VEGF/NRP regulation of P-Rex1 is Myc dependent. Indeed, we observed that VEGF was unable to induce P-Rex1 expression in the presence of Myc small hairpin RNA (shRNA) in PC3-S cells engineered to express NRP2 (Figure 7E). Expression of Myc is VEGF dependent based on the findings that downregulation of VEGF reduced Myc expression and addition of VEGF increased Myc expression (Figures 7B and 7E).

Myc is a regulator of prostate cancer and prostate-specific expression of a Myc transgene drives carcinogenesis in a stepwise fashion from PIN to invasive cancer (Ellwood-Yen et al., 2003). Myc-Cap cells were derived from this transgenic mouse model. Inhibition of Rac1 in Myc-CaP cells reduced their ability to form colonies in soft agar (Figure 7H). Moreover, downregulation of Rac1 or P-Rex-1 expression significantly increased tumor-free survival in vivo, establishing the important role of Rac1/P-Rex1 in Myc-induced tumorigenesis (Figures 7I–7K).

DISCUSSION

This study was predicated on the results from clinical trials concluding that bevacizumab and VEGF receptor tyrosine kinase inhibitors are not effective therapies for prostate cancer (Merino et al., 2011). It is widely assumed that these drugs target tumor angiogenesis (Merino et al., 2011) and, consequently, the poor response observed in these clinical trials could be considered in the context of angiogenesis and the role of angiogenesis in prostate cancer. In contrast to this prevailing idea, we focused on the hypothesis that VEGF signaling in tumor cells, especially cells with stem-like properties, is critical for tumor propagation and progression and that this signaling, mediated primarily by NRPs, is a prime target for therapy (Goel and Mercurio, 2013). Indeed, the results we report demonstrate that prostate cancer cells selected for their resistance to bevacizumab and sunitinib are enriched for stem cell properties and NRP signaling. Most importantly, we demonstrate that NRP signaling induces expression of P-Rex1, a Rac1 GEF, and that Rac1-mediated ERK activation is responsible for resistance to bevacizumab and sunitinib. These findings reveal a role for VEGF/NRP-mediated regulation of P-Rex1 in the biology of CSCs and resistance to therapy.

An intriguing aspect of our study is the "VEGF paradox." Specifically, we observed that resistance to VEGF-targeted therapy (bevacizumab and sunitinib) is mediated by an enhancement of VEGF/NRP signaling. In fact, prostate cancer cells treated with bevacizumab and sunitinib exhibit a marked increase in VEGF expression despite the fact that bevacizumab targets the interaction of VEGF with VEGFRs (Ferrara, 2005). Our interpretation of these data is that neither bevacizumab nor sunitinib is effective at targeting prostate cancer cells with stem cell properties and that the CSC population, which is characterized by autocrine VEGF/NRP signaling, is enriched by treatment with these drugs because they target primarily non-CSCs. This hypothesis is supported by several studies that have highlighted the importance of VEGF/NRP signaling in CSCs and discounted the contribution of VEGFRs (Goel and Mercurio, 2013). In light of our data that resistant cells show lack of VEGF2 surface expression, we propose that NRP2-mediated VEGF signaling is independent of its role as a co-receptor for VEGFRs. This hypothesis is consistent with previous reports that VEGF/NRP signaling can occur independently of VEGFRs (Goel and Mercurio, 2013; Cao et al., 2013). Moreover, our previous observation that NRP2 associates with the $\alpha 6\beta 1$ integrin and regulates CSC properties by activating focal adhesion kinase (FAK) (Goel et al., 2012, 2013)

Figure 4. Rac1 Inhibition Improves Sensitivity to VEGF-Targeted Therapy

(A) PC3-R cells were transfected with Rac1 shRNAs, and these cells were implanted in NSG mice. Once tumors reached ~100 mm^3 in volume, mice were treated with either control immunoglobulin G (Cont) or bevacizumab (Bev; 10 mg/kg, intraperitoneally [i.p.], twice weekly). Tumor volume was measured every third day.

(B) Control and treated PC3-R xenograft tumors were harvested, and apoptosis was analyzed using TUNEL staining. Scale bar, 10 μm.

(C and D) Six-week old PTEN$^{pc-/-}$ mice were injected i.p. with either mcr84 (10 mg/kg) or EHT1864 (10 mg/kg) twice weekly for 3 weeks. The GU tract was harvested, and total weight was measured. The prostate glands were separated and combined weight of all the lobes was measured. The prostate glands were digested, and LSC cells (Lin$^-$Sca$^+$CD49f^{high}) were isolated by fluorescence-activated cell sorting. The number of LSC cells is significantly reduced in mice treated with RACi or RACi+mcr84.

(E) H&E staining of prostate tumors from PTEN$^{pc-/-}$ mice described in (C). The percentage of prostate glands showing either PIN or well-differentiated adenocarcinoma (AdCa) was quantified as shown. Scale bar, 100 μm.

(F) Tumor sections of prostate tumors from PTEN$^{pc-/-}$ mice described in (C) were stained using the TUNEL reagent to detect apoptosis. Scale bar, 100 μm.

Error bars represent mean ± SD. Bev, bevacizumab; hIgG, control immunoglobulin G. See also Figures S3 and S4.

A

	C4-2S	C4-2R	PC3-S	PC3-R
	GEFs			
SOS1	1 (0.46)	0.99 (0.34)	1 (0.01)	1 (0.11)
P-Rex1	1 (0.065)	13.6 (2.3)	1 (0.046)	16.5 (0.48)
VAV1	1 (0.41)	1 (0.21)	1.02 (0.3)	1.09 (0.37)
VAV2	1 (0.29)	1 (0.21)	1 (0.07)	1 (0.36)
VAV3	1 (0.1)	1.2 (0.54)	1 (0.07)	1 (0.3)
TIAM1	1 (0.1)	6 (2.2)	1 (0..034)	8.7 (0.95)

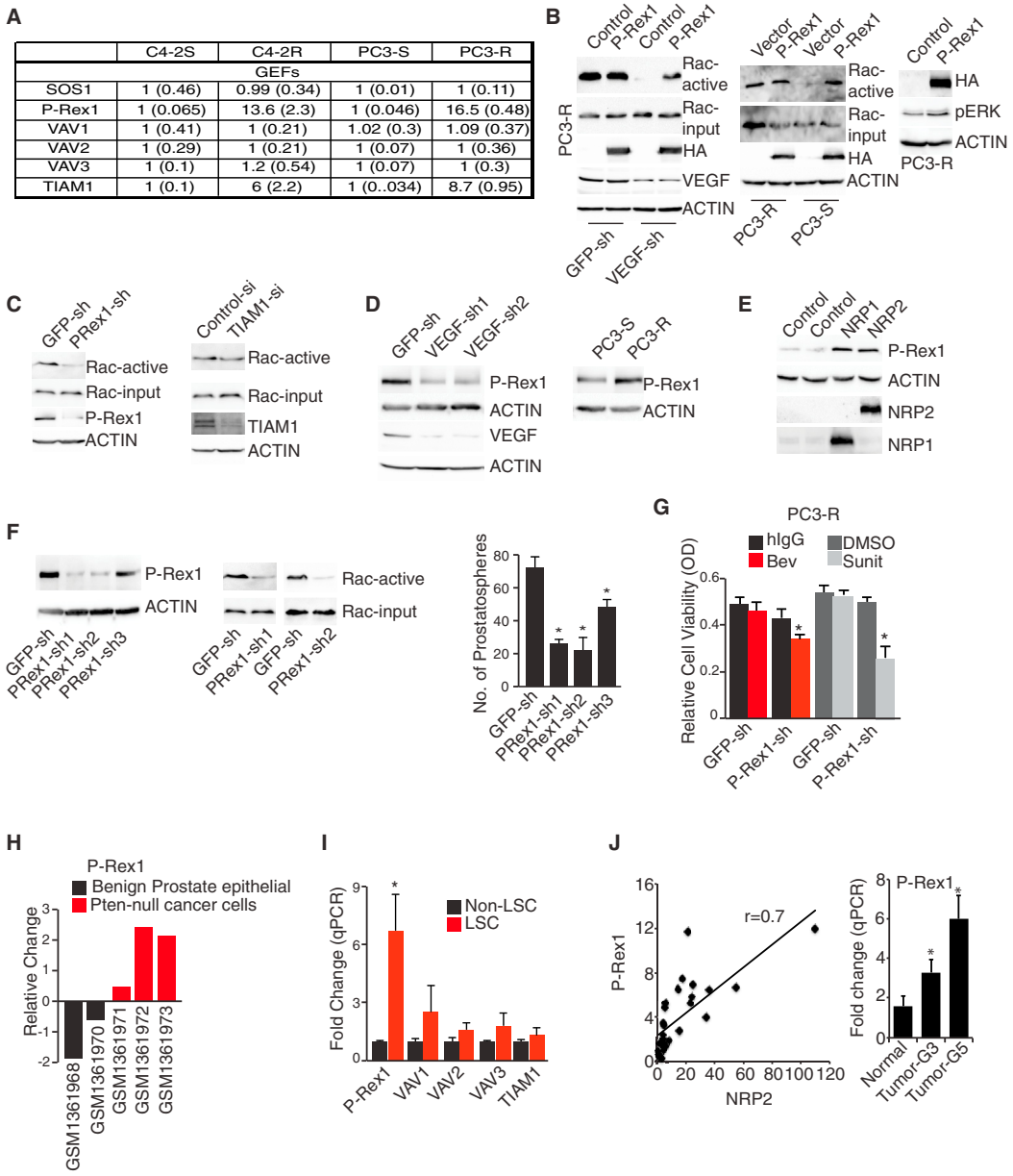

Figure 5. P-Rex1, a GEF, Promotes Rac1 Activation and Resistance to VEGF-Targeted Therapy

(A) The expression of Rac1 GEFs was compared in sensitive and resistant PC3 and C4-2 cell lines using qPCR. Table shows fold change in mRNA expression upon normalization with sensitive populations, which was set as 1.

(B) P-Rex1 was expressed in resistant PC3 cells in which VEGF expression had been diminished using shRNA and the effect on Rac activation was determined (left). P-Rex1 was expressed in resistant and sensitive PC3 cells and the effect on Rac activation was determined (middle). Right panels show the expression of HA-tagged P-Rex1 in PC3-R cells.

(C) Resistant PC3 cells were transfected with either P-Rex1 shRNA or TIAM1 siRNA, and the effect on Rac activation was determined.

(D) Protein extracts from resistant PC3 cells in which VEGF expression had been diminished using shRNA were immunoblotted with P-Rex1, VEGF, or actin antibodies.

(E) Either NRP1 or NRP2 was expressed in sensitive PC3 cells, and the effect on P-Rex1 expression was assessed by immunoblotting.

(F) Resistant PC3 cells were transfected with P-Rex1 shRNA, and the effect on prostatosphere formation and Rac1 activation was analyzed.

(G) Resistant PC3 cells expressing P-Rex1 shRNA were treated with bevacizumab (Bev; 1 mg/ml) or sunitinib (Sunit; 20 μM), and their proliferation was assayed. Beva, bevacizumab; hIgG, control immunoglobulin G.

(H) Expression of P-Rex1 was analyzed in a published dataset (GEO: GSE56469).

(I). Freshly harvested LSC cells from 9-week-old $PTEN^{pc-/-}$ mice were analyzed for expression of GEFs using qPCR.

(J) Expression of NRP2 and P-Rex1 mRNA was quantified by qPCR in microdissected sections from benign glands, as well as grade 3 and grade 5 prostate cancer specimens. A significant correlation (p value is 1×10^{-6}) in the expression of P-Rex1 and NRP2 was observed (r = 0.7).

Error bars represent mean ± SD.

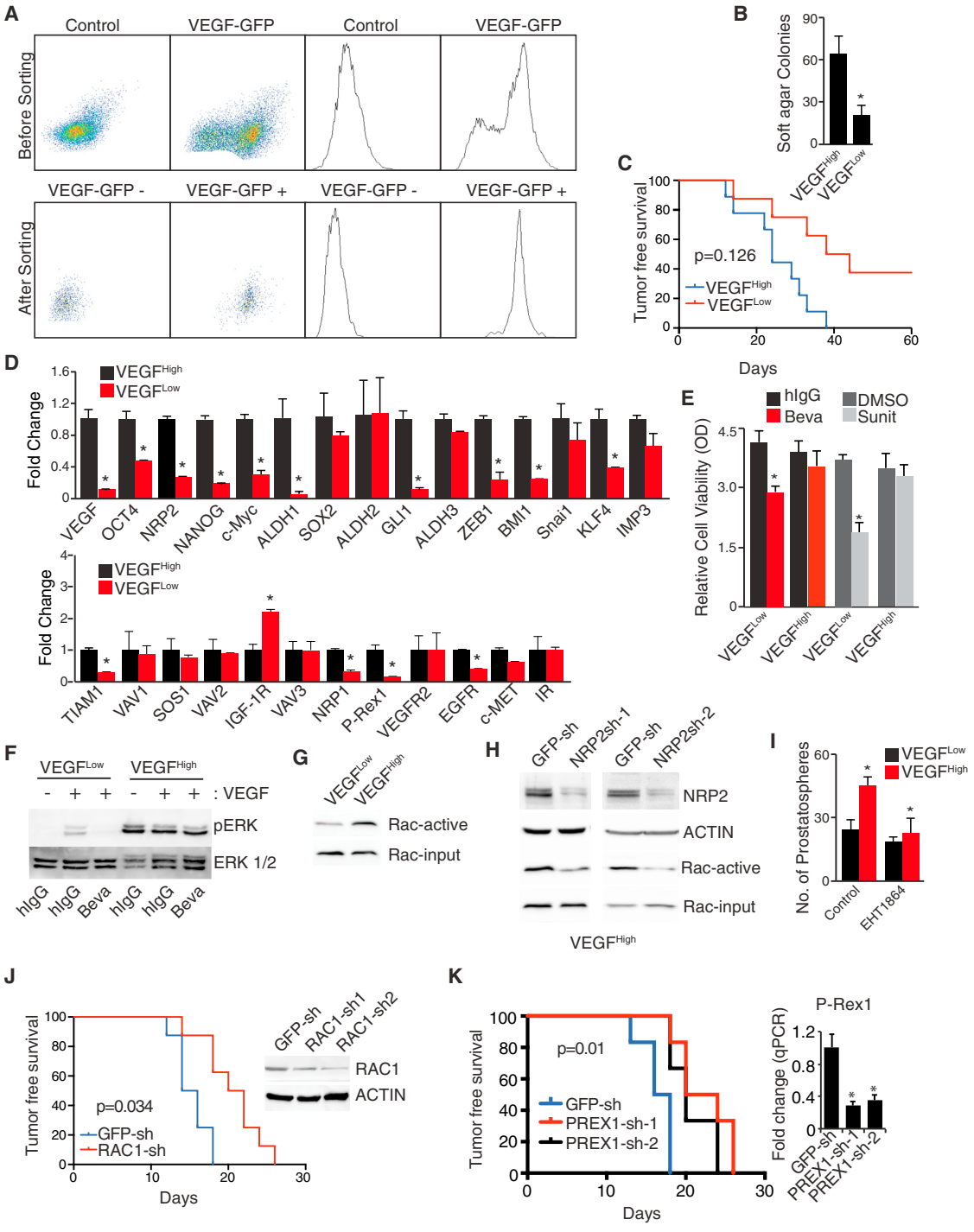

Figure 6. Rac1 Is Required for VEGF-Mediated Tumor Initiation

(A) PC3 cells were transfected with a GFP-expressing plasmid under control by the VEGF promoter and these cells were sorted based on their expression of GFP. The top panels show fluorescence-activated cell sorting (FACS) profile before GFP sorting, and the bottom panels show FACS profile after sorting.

(B) The ability of VEGF^{high} and VEGF^{low} cells to form colonies in soft agar was determined.

(C) VEGF^{high} and VEGF^{low} cells were implanted in NSG mice and tumor formation was detected by palpation.

(D) Expression of genes associated with stem cells and VEGF signaling was quantified by qPCR.

(E) VEGF^{high} and VEGF^{low} cells were incubated with bevacizumab (Bev; 1 mg/ml) or sunitinib (Sunit; 20 μM) for 72 hr, and their proliferation was assayed. Beva, bevacizumab; hIgG, control immunoglobulin G.

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provides a potential mechanism for VEGF signaling that is independent of VEGFRs because FAK is known to mediate ERK activation (Zhao and Guan, 2009) and is important for CSCs (Luo et al., 2009).

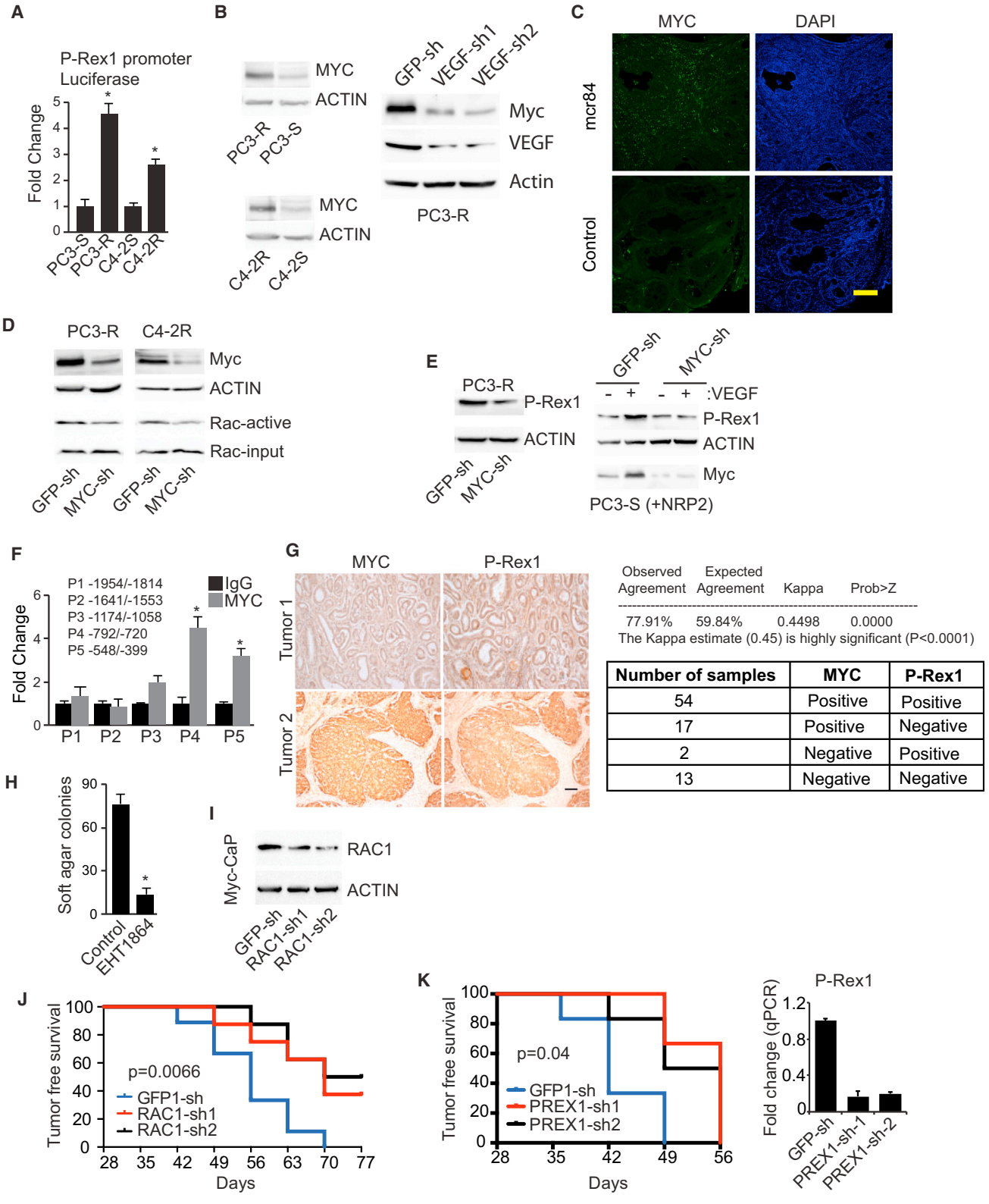
Our data reveal an unexpected role for P-Rex1 and Rac1 activation in the genesis of prostate CSCs and resistance to bevacizumab and sunitinib. P-Rex1 is quite interesting in this regard because its expression is low in normal prostate and elevated in metastatic disease (Qin et al., 2009). There is also evidence that P-Rex1 can promote metastasis in a xenograft model of prostate cancer (Qin et al., 2009). Although many studies have implicated Rac1 in migration, invasion, initiation, and growth of tumor cells, including prostate cancer (Bid et al., 2013; Baker et al., 2014), our results show that P-Rex1-mediated Rac1 activation is critical for the formation and function of prostate CSCs. This conclusion is demonstrated most rigorously by our observation that treatment of mice harboring $PTEN^{pc-/-}$ tumors with a Rac1 inhibitor significantly reduced the number of LSC cells, which have been characterized as CSCs in this transgenic model (Mulholland et al., 2009). Also, treatment of these mice with the Rac1 inhibitor reduced the frequency of tumor formation, consistent with a role for Rac1 in the function of CSCs. We also provide evidence that Rac1-mediated activation of ERK is responsible for resistance to bevacizumab and sunitinib.

We provide mechanistic insight into the regulation of P-Rex1 expression by identifying Myc as a regulator of P-Rex1 transcription in prostate CSCs. This finding is relevant because Myc is significantly elevated in prostate CSCs compared to non-CSCs (Civenni et al., 2013). Also, gene set enrichment analysis of two independent datasets revealed that Myc expression is associated with tumor cells enriched with an embryonic stem cell-like gene signature (Civenni et al., 2013). Our data also indicate that VEGF/NRP signaling contributes to the regulation of Myc expression and Myc-induction of P-Rex1. This conclusion is supported by the report that VEGF/VEGFR2 signaling induces Myc expression in breast cancer cells by a mechanism that involves Stat 3 (Zhao et al., 2015). Based on our data, however, VEGF induction of Myc appears to be independent of VEGFRs. In this direction, we reported that VEGF/NRP signaling activates FAK in CSCs. (Goel et al., 2012, 2013). This observation is interesting based on the report that FAK regulates Myc transcription in epidermal stem cells (Ridgway et al., 2012). It is also worth noting that epigenetic repression of P-Rex1 in non-aggressive prostate cancer cell lines has been observed (Wong et al., 2011). However, our initial experiments suggested that epigenetic regulation does not account for the marked increase in P-Rex1 mRNA expression in PC3-R cells compared to PC3-S (Figure S6A).

An important question that arises from our data is how P-Rex1-mediated Rac1 activation impacts the function of prostate CSCs and promotes resistance to therapy. We posit that P-Rex1/Rac1-mediated ERK activation sustains the expression of VEGF and NRP2 and the ability of VEGF/NRP2 signaling to enhance the expression of BMI-1 and other stem cell factors. In essence, we suggest that p-Rex1/Rac1-mediated ERK activation contributes to a positive feedback loop involving VEGF/NRP2 signaling that sustains stem cell properties in prostate cancer. In addition, our previous work demonstrated that VEGF/NRP2 signaling contributes to ERK-mediated induction of Gli1 and BMI-1 expression and that this pathway can feedback to sustain NRP2 expression (Goel et al., 2013). These findings should be discussed in the context of a recent report concluding that autocrine semaphorin 3C promotes the survival of glioma stem cells by activating Rac1/nuclear factor κB signaling (Man et al., 2014). In contrast to our results, however, they observed that semaphorin-3C-mediated Rac1 activation does not impact ERK activation or the expression of stem cell factors. We also analyzed the expression of semaphorin 3C and targets of nuclear factor κB signaling and found no difference between sensitive and resistant populations (Figures S6B–S6D). Clearly, the available data indicate that Rac1 can affect the function of CSCs by distinct mechanisms that may relate to the biology of specific cancers. It is also worth noting that both semaphorin 3C and VEGF are ligands for NRP2, and an important aspect of our work is that we implicate VEGF-mediated activation of P-Rex1/Rac1 in resistance to bevacizumab, which has significant therapeutic implications. Interestingly, in this context, our analysis of gene profiling of metastatic colon cancer patients treated with bevacizumab revealed that high P-Rex1 or Myc expression is a significant predictor of poor progression-free survival (Figure S6E) (Pentheroudakis et al., 2014). Also, the analysis of gene expression in human glioblastoma xenografts treated with bevacizumab indicated increased expression of P-Rex1 and NRP2 (Figure S6F). Unfortunately, it is not possible to perform a similar analysis of prostate cancer patients treated with either bevacizumab or sunitinib (Michaelson et al., 2014; Kelly et al., 2012) because tumor specimens were not collected as an endpoint in these clinical trials (W.K. Kelly and M.D. Michaelson, personal communication).

Our data raise the exciting possibility that bevacizumab or VEGFR-targeted therapy in prostate cancer could be efficacious if it were combined with targeted inhibition of P-Rex1/Rac1. This possibility is supported by the data presented in Figures 4A and 6K. It is also timely and significant because there are few therapeutic options available for men with aggressive prostate cancer, which is enriched with tumor cells with a stem-like phenotype (Chen et al., 2013). Potent Rac1 inhibitors are available (Montalvo-Ortiz et al., 2012), but some concern is noted with their potential side effects as indicated by the reduced weight

(F) $VEGF^{high}$ and $VEGF^{low}$ cells were serum-deprived overnight and stimulated with VEGF (50 ng/ml) for 30 min in the presence or absence of bevacizumab (5 mg/ml). The activation of ERK was analyzed by immunoblotting using a phospho-specific antibody.
(G) Rac1 activation was compared in $VEGF^{high}$ and $VEGF^{low}$ cells.
(H) NRP2 expression in $VEGF^{high}$ cells was downregulated using shRNA, and Rac1 activation was assayed.
(I) Prostatosphere formation by $VEGF^{high}$ cells in the presence or absence of EHT1864 was quantified.
(J and K) $VEGF^{high}$ cells were transfected with shRNAs targeting either Rac1 (J) or P-Rex1 (K), and these cells were implanted in NSG mice. Tumor formation was detected by palpation.
Error bars represent mean ± SD.



Number of samples	MYC	P-Rex1
54	Positive	Positive
17	Positive	Negative
2	Negative	Positive
13	Negative	Negative

Figure 7. Myc Regulates PREX1 Transcription in Resistant Cells

(A) A luciferase reporter construct containing the P-Rex1 promoter was expressed in sensitive and resistant PC3 and C4-2 cells, and luciferase activity was measured and normalized to Renilla.

(legend continued on next page)

of the GU tract in response to EHT1864 (Figure 4D). Targeting P-Rex1, however, may be more feasible based on our data. Nonetheless, our data demonstrate that P-Rex1/Rac1 inhibition reduces stem cell properties and renders tumor cells more sensitive to VEGF-targeted therapies.

EXPERIMENTAL PROCEDURES

Animal Studies

All mouse experiments were performed following a protocol approved by the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School.

Cell Lines

PC3 (ATCC), C4-2 (UroCor), and MyC-CaP (provided by Dr. Charles L. Sawyers, Memorial Sloan-Kettering Cancer Center, New York, NY) were used. shRNA clones from the RNAi Consortium library were obtained from RNAi core, University of Massachusetts Medical School.

Cell-Based Assays

The chemosensitivity of prostate cancer cells was determined using a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay (Mosmann, 1983). The assay was performed 72 hr after treatment. Fluorescence-activated cell sorting was used to isolate cells based on their surface expression of CD44, CD24, and α6 and β4 integrins. The detailed procedure for isolating LSC cells from $PTEN^{pc-/-}$ mice using lineage markers (CD31, CD45, and Ter119), Sca-1, and CD49f is described previously (Mulholland et al., 2009; Lawson et al., 2007).

Isolation of Human Prostate Tumor Cells and Laser Capture Microscopy

Human prostate tumor tissue was obtained from UMASS Cancer Center Tissue Bank in compliance with the institutional review board of the University of Massachusetts Medical School. The discarded but freshly resected, prostate tumors were digested with collagenase at 37°C, and epithelial cells were isolated using an EpCaM antibody. Frozen sections were microdissected by laser capture microscopy (Arcturus PixCell 2) as described elsewhere (Goel et al., 2012) to obtain pure populations of tumor cells of defined Gleason grades. RNA was isolated from these microdissected samples using the RNeasy kit (QIAGEN), and cDNA was prepared using Superscript II reverse transcriptase (Invitrogen). Quantitative real-time PCR was done using the TaqMan assay kit (Applied Biosystems).

Promoter Activity and ChIP Assays

Prostate cancer cells were transfected with the P-Rex1 promoter luciferase construct (−2021/+3) and Renilla luciferase construct to normalize for transfection efficiency. Relative light units were calculated upon normalization with Renilla luciferase activity. ChIP assays were performed according to our published protocol (Goel et al., 2012). All ChIP experiments were repeated at least two times. The sequence of primers used to amplify the P-Rex1 promoter is provided in Figure S6G.

Statistics

Unless otherwise cited, all values are presented as the mean ± SD. For the Student's t test, comparisons between two groups were performed using two-tailed, assuming equal variance among groups. A p value less than 0.05 was considered significant. The correlation of Myc and P-Rex1 expression in human prostate cancer specimens was done using kappa statistics. The kappa estimate was tested against a null hypothesis of kappa = 0.0. For tumor-free survival xenograft experiments, the comparison between two curves was done using the log-rank (Mantel-Cox) test. All experiments were repeated at least twice with the exception of experiments involving the culture of primary tumor cells, and data from one representative experiment are shown.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.02.016.

AUTHOR CONTRIBUTIONS

H.L.G. designed, executed, and analyzed experiments and wrote the manuscript. B.P. contributed to the experiments. C.W.V.K. provided NRP2 inhibitory antibody and performed plate-based inhibition assays. L.D.S. and D.L.G. provided the NSG mice. R.A.B. provided mcr84 antibody. A.M.M. supervised the study and wrote the manuscript together with H.L.G.

CONFLICTS OF INTEREST

D.L.G. is a consultant for the Jackson Laboratory. R.A.B. has a commercial research grant from Peregrine Pharmaceuticals as well as other commercial research support from Affitech and is a consultant/advisory board member of Peregrine Pharmaceuticals.

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(B) Myc expression was compared between sensitive and resistant PC3 and C4-2 cells by immunoblotting (left). VEGF expression was downregulated in PC3-R cells using shRNAs, and the effect on Myc expression activation was determined by immunoblotting (right).
(C) Six-week-old $PTEN^{pc-/-}$ mice were injected (i.p.) with mcr84 (10 mg/kg) twice weekly for 3 weeks. The prostate glands were harvested and immunostained using a myc antibody.
(D) Myc expression was downregulated in resistant PC3 and C4-2 cells using shRNA, and the effect on Rac1 activation was determined.
(E) Myc expression was downregulated in PC3-R cells using shRNA, and the effect on P-Rex1 expression was determined (left). Right: NRP2-expressing PC3-S cells were transfected with either GFP-sh or Myc-sh and stimulated with VEGF (50 ng/ml) for 24 hr, and the effect on P-Rex1 and Myc expression was measured.
(F) ChIP was performed using a Myc antibody and regions of the PREX1 promoter that bound Myc were identified and quantified by qPCR.
(G) The expression of Myc and P-Rex1 was analyzed in human prostate cancer specimens by immunohistochemistry. A significant correlation of their expression was detected. The kappa estimate (0.45) is highly significant ($p < 0.0001$), and it was tested against a null hypothesis of kappa = 0.0. Scale bar, 100 μm.
(H) The ability of Myc-CaP cells to form colonies in soft agar in the presence or absence of EHT1864 was determined.
(I and J) Myc-CaP cells were transfected with two different Rac1 shRNAs, and the effect on Rac1 expression was detected by immunoblotting (I). These cells were implanted into NSG mice, and tumor onset was determined by palpation (J).
(K). Myc-CaP cells were transfected with two different P-Rex1 shRNAs, and the effect on P-Rex1 expression was quantified by qPCR (right). These cells were implanted into NSG mice, and tumor onset was determined by palpation (left).
Error bars represent mean ± SD. Bev, bevacizumab; hIgG, control immunoglobulin G. See also Figures S5–S7.

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CANCER DISCOVERY

VEGF/Neuropilin-2 Regulation of Bmi-1 and Consequent Repression of IGF-IR Define a Novel Mechanism of Aggressive Prostate Cancer

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RESEARCH ARTICLE

VEGF/Neuropilin-2 Regulation of Bmi-1 and Consequent Repression of IGF-IR Define a Novel Mechanism of Aggressive Prostate Cancer

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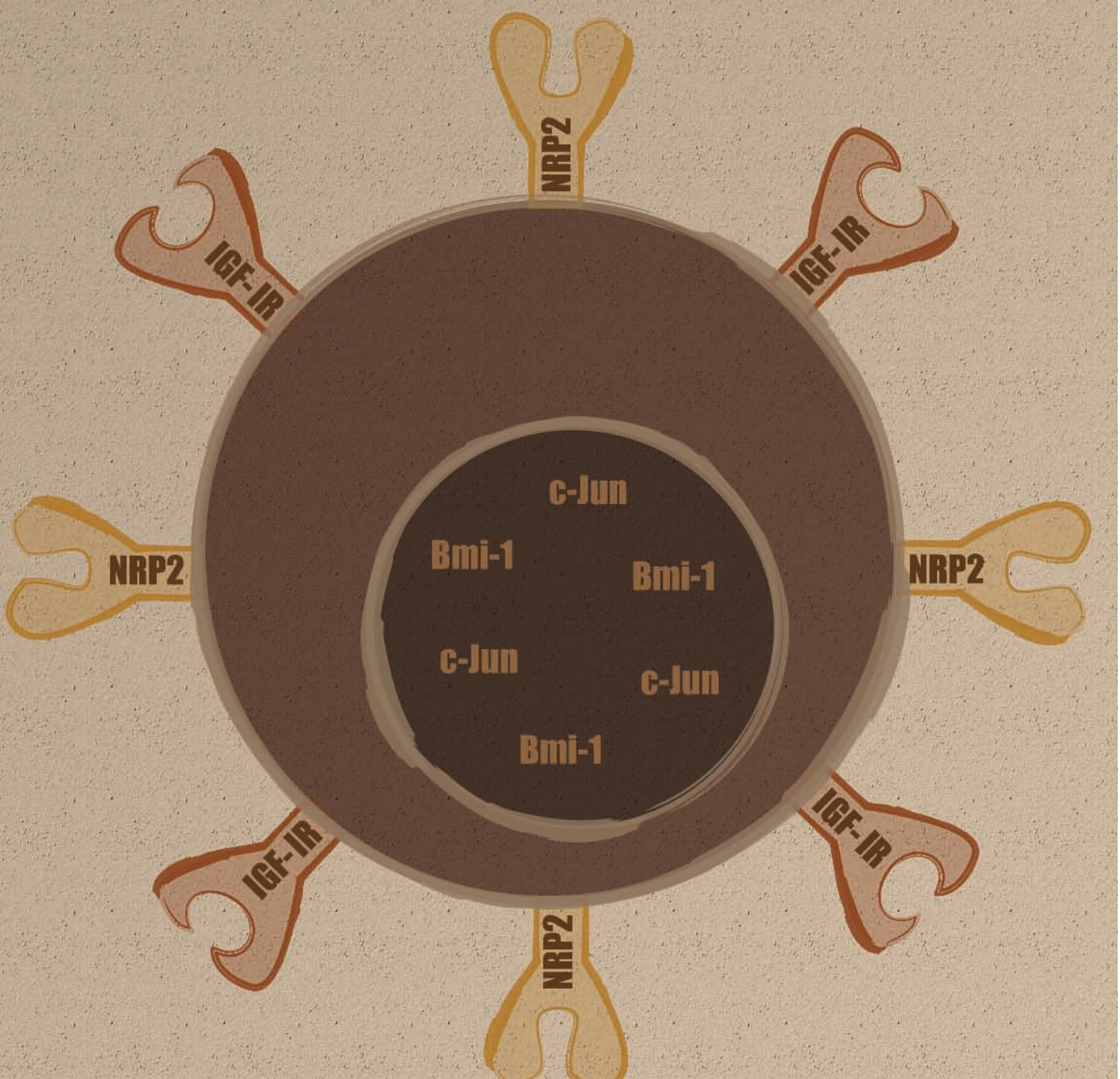

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ABSTRACT We show that the VEGF receptor neuropilin-2 (NRP2) is associated with high-grade, PTEN-null prostate cancer and that its expression in tumor cells is induced by PTEN loss as a consequence of c-Jun activation. VEGF/NRP2 signaling represses insulin-like growth factor-1 receptor (IGF-IR) expression and signaling, and the mechanism involves Bmi-1–mediated transcriptional repression of the *IGF-IR*. This mechanism has significant functional and therapeutic implications that were evaluated. IGF-IR expression positively correlates with PTEN and inversely correlates with NRP2 in prostate tumors. NRP2 is a robust biomarker for predicting response to IGF-IR therapy because prostate carcinomas that express NRP2 exhibit low levels of IGF-IR. Conversely, targeting NRP2 is only modestly effective because NRP2 inhibition induces compensatory IGF-IR signaling. Inhibition of both NRP2 and IGF-IR, however, completely blocks tumor growth *in vivo*.

SIGNIFICANCE: These results identify a causal role for NRP2 and VEGF/NRP2 signaling in the behavior of aggressive prostate cancers by a mechanism that involves regulation of Bmi-1, a transcriptional repressor implicated in the etiology of prostate cancer induced by loss of PTEN function, and the repression of the IGF-IR. The therapeutic implications are significant because combined inhibition of NRP2 and IGF-IR overcomes the resistance induced by targeting each receptor individually. *Cancer Discov; 2(10); 906–21. ©2012 AACR.*

INTRODUCTION

Understanding mechanisms that drive the initiation and progression of prostate cancer is essential to improving the clinical management of this disease, which is the most common cancer in men in the United States and Western Europe (1). Although early-stage prostate cancer can be treated reasonably well clinically, advanced forms of this disease are very aggressive and difficult to manage with existing therapies. As a consequence, these tumors are associated with a high degree of morbidity and mortality. Our approach to understanding the biology of aggressive prostate cancer is rooted in the hypothesis that tumor cells express receptors for VEGF and that VEGF signaling in tumor cells contributes to tumor formation and progression (2–5). Surprisingly, however, little is known about the expression and function of VEGF receptors on prostate carcinoma cells and their contribution to this disease. This problem is significant because these receptors and the signaling pathways that they regulate are prime targets for therapeutic intervention.

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Note: Supplementary data for this article are available at Cancer Discovery Online (http://cancerdiscovery.aacrjournals.org/).

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The neuropilins (NRP) are one class of VEGF receptors that are particularly interesting with respect to cancer biology. NRP1 and NRP2 were identified initially as neuronal receptors for semaphorins, which are axon guidance factors that function primarily in the developing nervous system (6, 7). The seminal finding by Soker and colleagues that neuropilins can also function as VEGF receptors and that they are expressed on endothelial and tumor cells launched studies aimed at understanding their functional contribution to angiogenesis and tumor biology (8). Neuropilins have the ability to interact with and modulate the function of tyrosine kinase VEGF receptors (VEGFR1 and VEGFR2) as well as other receptors (9, 10). There is also evidence that neuropilins can function independently of other receptors (11) and that they are valid targets for therapeutic inhibition of angiogenesis and cancer (12–14).

This study shows that the expression of NRP2 in prostate cancer cells is induced by PTEN deletion and that its expression correlates with Gleason grade. Given that the inactivation of PTEN is one of the most common genetic lesions in prostate cancer and its frequency increases with more aggressive disease (15), our discovery led to the hypothesis that VEGF/NRP2 signaling in tumor cells has a key role in prostate carcinogenesis. Indeed, the data obtained reveal that VEGF/NRP2 signaling contributes to the expression of Bmi-1, a Polycomb group transcriptional repressor that has been implicated in the etiology of prostate cancer induced by PTEN deletion (16). We also discovered that Bmi-1 represses transcription of the *insulin-like growth factor-I receptor (IGF-IR)*, which is relevant because IGF-IR signaling contributes to tumor growth (17) and the IGF-IR is a potential therapeutic target for prostate cancer (18). We conclude from our data that aggressive prostate cancer cells exhibit VEGF/NRP2 signaling, Bmi-1 expression, and reduced IGF-IR expression. The functional and therapeutic implications of these findings were evaluated, and the results highlight a novel role for NRP2 as a biomarker for predicting response to IGF-IR therapy and as a therapeutic target itself in combination with IGF-IR inhibition.

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RESULTS

NRP2 Is Induced in Prostate Cancer as a Consequence of PTEN Loss and JNK1/c-Jun Activation

Although aggressive prostate cancer is characterized by elevated VEGF expression in tumor cells (19, 20), therapeutic approaches based on VEGF inhibition have been largely unsuccessful. Bevacizumab, a VEGF antibody, did not have an impact on patients with metastatic, castration-resistant prostate cancer, and the trial was halted because it did not meet predefined response goals (21). Bevacizumab, however, does not inhibit the interaction of VEGF with the VEGF receptor NRP2 (22), which is noteworthy because we observed that NRP2 expression is induced in prostate cancer and that it is correlated with Gleason grade (Fig. 1A and B). Specifically, there is no significant NRP2 expression in normal, immortalized prostate cell lines, normal prostatic epithelium, or prostatic intraepithelial neoplasia [(PIN) Fig. 1A–C]. The expression of NRP2 in prostate cancer cell lines correlates with their invasive potential (Fig. 1A; Supplementary Fig. S1A). Robust NRP2 expression is seen in high-grade prostate cancer (Gleason grade 5) and metastases compared with low-grade prostate cancer (Gleason grade 3; Fig. 1B and C; Supplementary Fig. S1B–S1D). Importantly, NRP2 expression in prostate cancer is correlated significantly with grade ($P < 0.001$) and clinical outcome ($P = 0.047$; Supplementary Fig. S2A–S2D). Prostate carcinoma cells also express VEGFR2 but lack expression of VEGFR1 (Supplementary Fig. S2E; refs. 23, 24).

Given that PTEN loss has a causative role in the genesis of aggressive prostate cancer and correlates with Gleason grade (15, 25), we assessed the association of NRP2 expression with PTEN loss and observed a striking correlation. Relatively high NRP2 expression is seen in high Gleason grade tumors and metastases, which exhibit frequent PTEN loss (Fig. 1B; refs. 15, 25). Interestingly, however, there is heterogeneity in NRP2 expression in Gleason grade 3 prostate cancer specimens, in which approximately 12.5% of the cases expressed high levels of NRP2 (expression levels 4 and 5; Fig. 1B; Supplementary Fig. S2A–S2D). Gleason grade 3 tumors that harbored a PTEN deletion were positive for NRP2 expression, but those tumors that expressed PTEN were negative for NRP2 (Fig. 2A). These data are consistent with the finding that PTEN loss

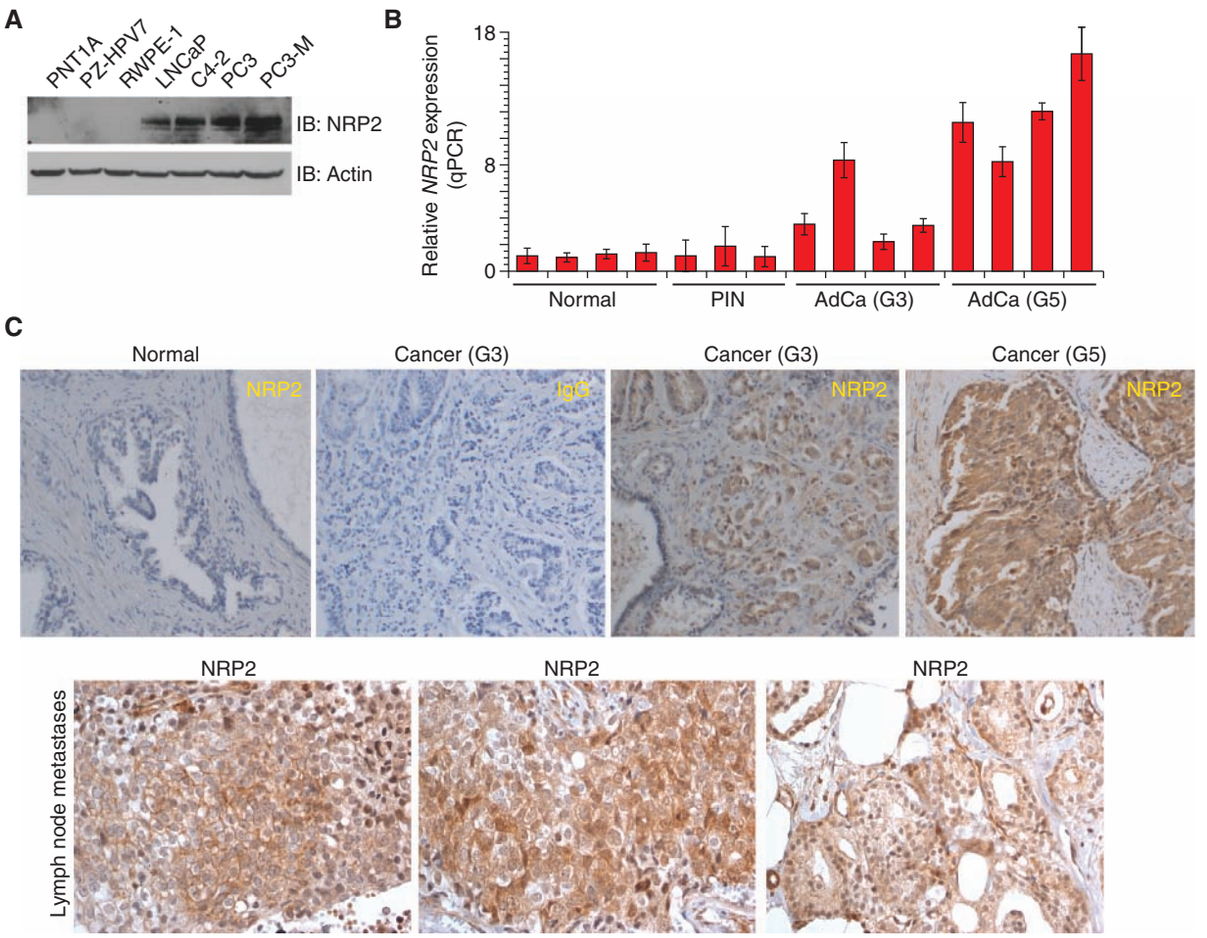

Figure 1. NRP2 expression is associated with prostate cancer progression. **A,** normal (PNT1A, PZ-HPV7, and RWPE-1) or prostate cancer (LNCaP, C4-2, PC3, and PC3-M) cell lines were immunoblotted (IB) using antibodies to NRP2 or actin. **B,** expression of *NRP2* mRNA was quantified by quantitative PCR (qPCR) in microdissected sections from normal glands, PIN, grade 3 and 5 prostate cancer specimens. **C,** specimens of normal prostate gland, Gleason grade 3 and 5 carcinoma, and lymph node metastases were immunostained with an NRP2 antibody or a control immunoglobulin G (IgG).

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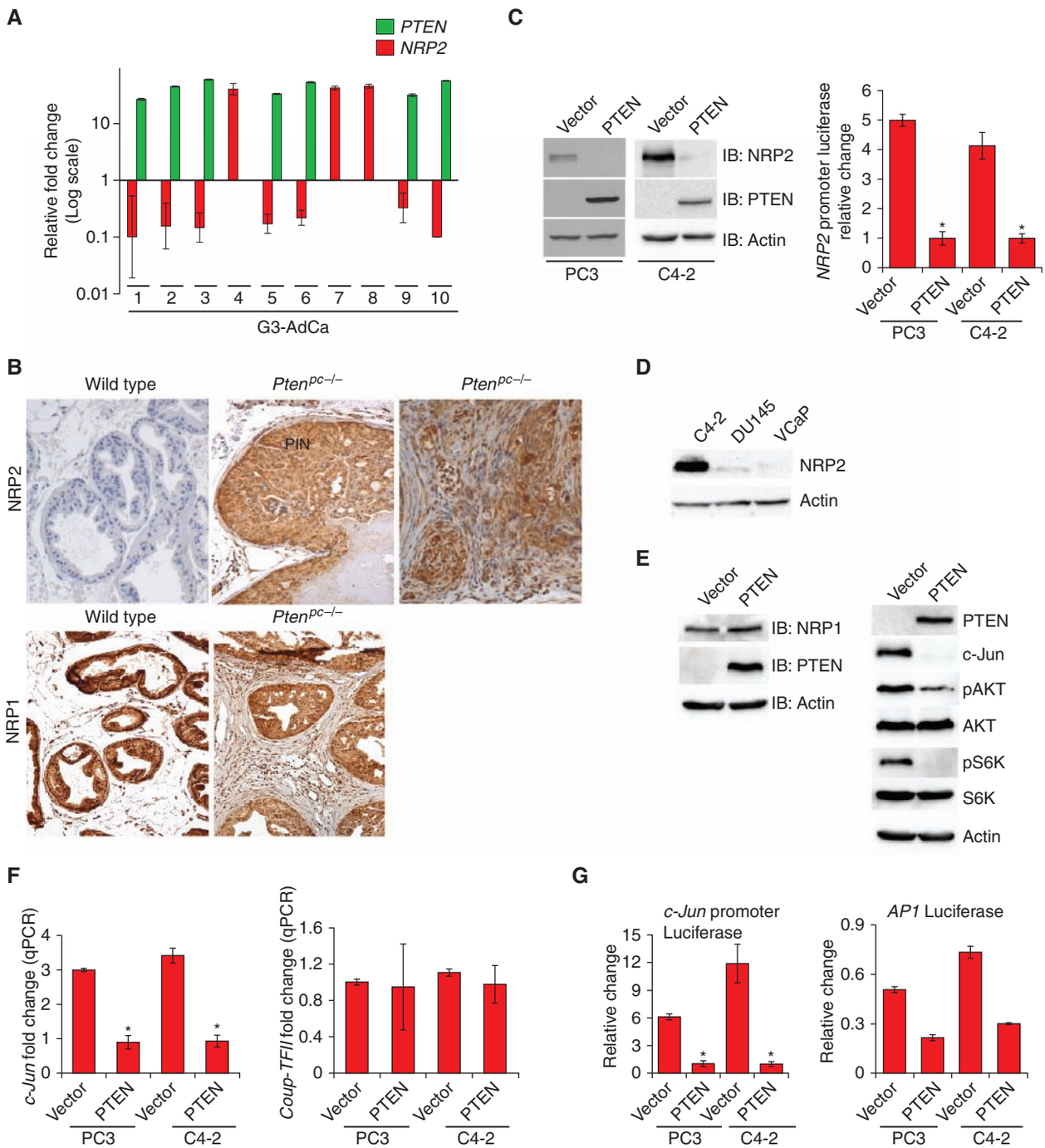

Figure 2. PTEN loss induces *NRP2* and c-Jun. **A,** microdissected specimens of tumor cells from Gleason grade 3 prostate cancer specimens were analyzed for *NRP2* and *PTEN* expression by quantitative PCR (qPCR). **B,** wild-type or *Pten*$^{pc-/-}$ prostates [PIN (6 weeks) and carcinoma (20 weeks)] were stained with either an NRP2 or an NRP1 antibody. NRP2 expression was not detected in the wild-type prostate ($n = 4$), but there is significant NRP2 expression in PIN lesions and carcinomas of *Pten*$^{pc-/-}$ mice ($n = 4$). In contrast, NRP1 was expressed in both normal prostate epithelium and carcinomas. **C,** PC3 or C4-2 cells were transfected with either a PTEN-expressing vector or vector control, and extracts were immunoblotted (IB) for PTEN, NRP2, and actin (left blot). These cells were also transfected with a luciferase reporter construct containing the *NRP2* promoter. Luciferase activity was measured and normalized to *Renilla* (right graph). *, $P < 0.05$. **D,** extracts from C4-2, VCaP, and DU145 cells were immunoblotted for NRP2 and actin. **E,** PC3 cells were transfected with either a PTEN-expressing vector or vector control, and extracts were immunoblotted for PTEN, NRP1, and actin (left blot) or for key signaling molecules (right blot). **F,** PC3 or C4-2 cells were transfected with either a PTEN-expressing vector or vector control, and the expression of *c-Jun* (left graph) and *Coup-TFII* mRNA (right graph) was quantified by qPCR. *, $P < 0.05$. **G,** PC3 or C4-2 cells expressing either PTEN or vector control were transfected with reporter constructs containing either the *c-Jun* promoter or *AP1* reporter. Luciferase activity was measured and normalized to *Renilla*. *, $P < 0.05$.

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occurs in approximately 15% to 20% of low-grade prostate cancer (15, 25). The association between PTEN loss and NRP2 expression was substantiated by an analysis of prostate tumors in *Pten*$^{loxp/loxp}$; *PB-Cre*$^{+}$ (referred to as *Pten*$^{pc-/-}$) mice, which are invasive and aggressive (26). These tumors display relatively high NRP2 expression compared with the mouse prostatic epithelium (Fig. 2B). NRP2 expression is also high in PIN lesions in the *Pten*$^{pc-/-}$ mice, consistent with the hypothesis that PTEN loss induces NRP2 (Fig. 2B). This consequence of PTEN loss is specific to NRP2 because NRP1 is expressed in both normal prostate epithelium and *PTEN*$^{pc-/-}$ tumors (Fig. 2B; Supplementary Fig. S3A). These results imply that PTEN represses NRP2 but not NRP1 expression. To test this hypothesis, PTEN was expressed in PC3 and C4-2 cells, which are PTEN-null and express NRP2. PTEN expression ablated NRP2 expression and the activity of a NRP2 promoter reporter construct but it had no effect on NRP1 expression (Fig. 2C–E). These results indicate that NRP2 is associated with aggressive, PTEN-null prostate cancer. Importantly, NRP2 expression is not a function of tumor differentiation because of the heterogeneity of its expression in grade 3 tumors, which are all differentiated, and our observation that NRP2 is not expressed in VCaP cells, which are poorly differentiated but PTEN-positive (Fig. 2D). DU145 cells, which are PTEN-positive, also lack NRP2 (Fig. 2D). Additional evidence to support the conclusion that PTEN loss or inactivation induces NRP2 was obtained by evaluating AKT activation because it is a read-out of PTEN deletion and mutation (27). Indeed, a significant correlation between phosphorylated AKT (p-AKT) and NRP2 expression was observed in prostate tumors (Supplementary Fig. S3B and S3C).

To investigate the mechanism behind PTEN-mediated NRP2 suppression, we analyzed the role of c-Jun because it is activated upon PTEN deletion in prostate tumors and contributes to tumorigenesis (28, 29). A significant downregulation of c-Jun occurred upon PTEN expression in PC3 and C4-2 cells (Fig. 2E and F), and PTEN expression inhibited the activity of a c-Jun promoter and AP1 reporter constructs significantly (Fig. 2G). However, PTEN expression did not affect Coup-TFII, a transcription factor previously shown to regulate NRP2 in lymphatic vessel development (Fig. 2F; refs. 30). We established the role of c-Jun in the regulation of NRP2 using c-Jun short hairpin RNA (shRNA) and TAM67 (a dominant-negative c-Jun). Expression of either c-Jun shRNAs or TAM67 reduced NRP2 protein and mRNA levels, as well as NRP2 promoter activity (Fig. 3A and B). Also, an increase in NRP2 promoter activity upon c-Jun overexpression was observed in PC3 and C4-2 cells (Fig. 3C). These results indicate that PTEN represses NRP2 expression by inhibiting c-Jun. We assessed this hypothesis by showing that c-Jun expression rescued NRP2 expression in PC3 and C4-2 cells that had been transfected with a PTEN construct (Fig. 3D). Moreover, our analysis of recently published gene expression data derived from PTEN induction in *Pten*$^{-/-}$ cells (29) revealed an inverse correlation between c-Jun and PTEN and between NRP2 and PTEN expression (Fig. 3E).

To investigate the regulation of NRP2 by c-Jun more rigorously, we carried out chromatin immunoprecipitation (ChIP) and observed that c-Jun binds directly to distinct sites in the NRP2 promoter (Fig. 3F and G). More specifically, the NRP2 promoter contains 2 AP1-binding sites located within NP3 and NP7 (Fig. 3F) and ChIP confirmed binding to these sites (Fig. 3G). In addition, mutating these sites inhibited NRP2 promoter activity (Supplementary Fig. S4A). As further proof that PTEN represses NRP2 expression by a c-Jun–dependent mechanism, we observed that PTEN significantly inhibited the binding of c-Jun to the NRP2 promoter (Fig. 3H). Given that c-jun-NH_2-kinases (JNK) are the upstream kinases that regulate c-Jun activity (31), we found that either a dominant-negative JNK or a JNK1 shRNA, but not a JNK2 shRNA, inhibited NRP2 expression (Supplementary Fig. S4B–S4D).

NRP2 Represses IGF-IR Expression and Inhibits IGF-IR Signaling

To evaluate the role of NRP2 in prostate tumorigenesis, we generated PC3 cells with diminished NRP2 expression using shRNAs. Loss of NRP2 had no effect on morphology or proliferation on tissue culture plates (data not shown), but it did reduce the ability of these cells to grow in soft agar (Fig. 4A, left). The NRP2-depleted cells also grew more slowly than control cells when implanted subcutaneously in mice (Fig. 4A, right). Although significant, the impact of NRP2 loss on growth in soft agar and tumor formation *in vivo* was modest, suggesting a potential compensatory mechanism. In this context, we observed a marked increase in *IGF-IR* mRNA expression concomitant with NRP2 loss but no change in the expression of either *EGF receptor (EGFR)* or *insulin receptor (IR)* mRNA expression (Fig. 4B). This effect of NRP2 loss on the induction of IGF-IR expression was also evident at the protein level in both PC3 and C4-2 cells (Fig. 4C). This effect is specific to NRP2 because no change in IGF-IR expression was observed upon NRP1 loss (Fig. 4D). The repression of IGF-IR expression by NRP2 in PC3 cells was maintained *in vivo* as evidenced by the observation that loss of NRP2 induced a significant increase in IGF-IR in 3 independent xenograft tumors (Fig. 4E). Also, immunostaining of human prostate cancer specimens revealed a positive correlation between p-AKT and NRP2 expression but a negative correlation between NRP2 and IGF-IR (Supplementary Fig. S3B and S3C). The possibility existed that FOXO transcription factors contribute to the regulation of IGF-IR expression based on the recent report that phosphoinositide 3-kinase activation represses receptor tyrosine kinases including the IGF-IR by feedback inhibition through inactivation of FOXO transcription factors (32). However, no change in FOXO activation was observed upon NRP2 depletion, and downregulation of FOXO factors did not affect the induction of IGF-IR expression caused by NRP2 (Supplementary Fig. S5A–S5C). Thus, the mechanism we propose appears to be distinct from this interesting study because inactivation of FOXO factors affected a number of receptors including the insulin receptor, EGFR, and c-MET. In contrast, NRP2 represses only the IGF-IR (Fig. 4B).

To investigate the role of NRP2 on IGF-IR signaling and function further, we expressed NRP2 in p69 noncancerous prostate epithelial cells, which express high levels of IGF-IR and less c-Jun than more aggressive cell lines (33). p69 cells are NRP2-null, and expression of NRP2 reduced IGF-IR

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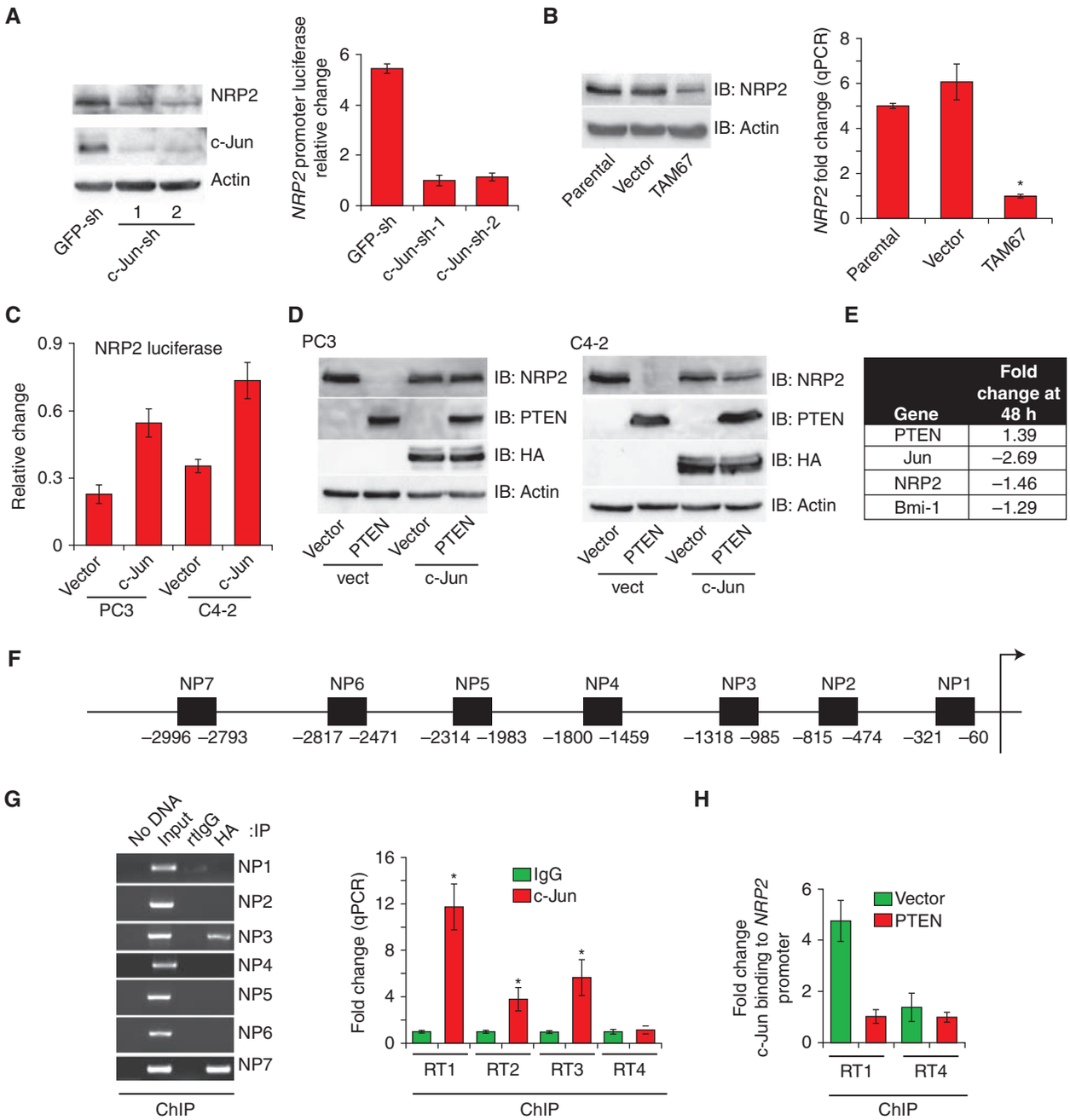

Figure 3. c-Jun regulates NRP2 expression. **A,** PC3 cells were transfected with either a GFP-shRNA or 2 independent c-Jun-shRNAs (1 and 2), and extracts were immunoblotted for NRP2, c-Jun, and actin (left blot). An *NRP2* promoter reporter construct was expressed in these cells, and luciferase activity was normalized to *Renilla* (right graph). **B,** PC3 cells were transfected with either TAM67 (a dominant-negative c-Jun construct) or vector control. Extracts were immunoblotted (IB) for NRP2 and actin (left blot), and *NRP2* mRNA expression was quantified by quantitative PCR (qPCR; right graph). *, $P < 0.05$. **C,** a c-Jun expression vector or control was expressed in either PC3 or C4-2 cells along with a *NRP2* promoter reporter construct, and luciferase activity was normalized to *Renilla*. **D,** PC3 or C4-2 cells expressing either PTEN or vector control were transfected with c-Jun (HA-tagged), and protein extracts were immunoblotted for NRP2, PTEN, hemagglutinin (HA), and actin. **E,** variation in the expression of PTEN, c-Jun, NRP2, and Bmi-1 upon PTEN induction (48 hours) in *Pten*$^{-/-}$ cells as described in Results. **F,** schematic representation of the *NRP2* promoter. The arrow indicates the transcriptional start site. Highlighted boxes (NP1-7) represent the primers used to amplify the ChIP DNA using semiquantitative PCR in **G. G,** PC3 cells were transfected with an HA-c-Jun construct and ChIP was conducted using an HA antibody or rat IgG to identify c-Jun–binding sites on the *NRP2* promoter. The precipitated DNA was amplified by PCR using primers specific for NP regions 1 to 7 (left gel), and the results were quantified by qPCR (right graph). *, $P < 0.05$. The RT1–4 primers used for qPCR are described in Supplementary Fig. S8. **H,** the effect of PTEN expression on c-Jun binding to the *NRP2* promoter was assessed by ChIP and quantified by qPCR.

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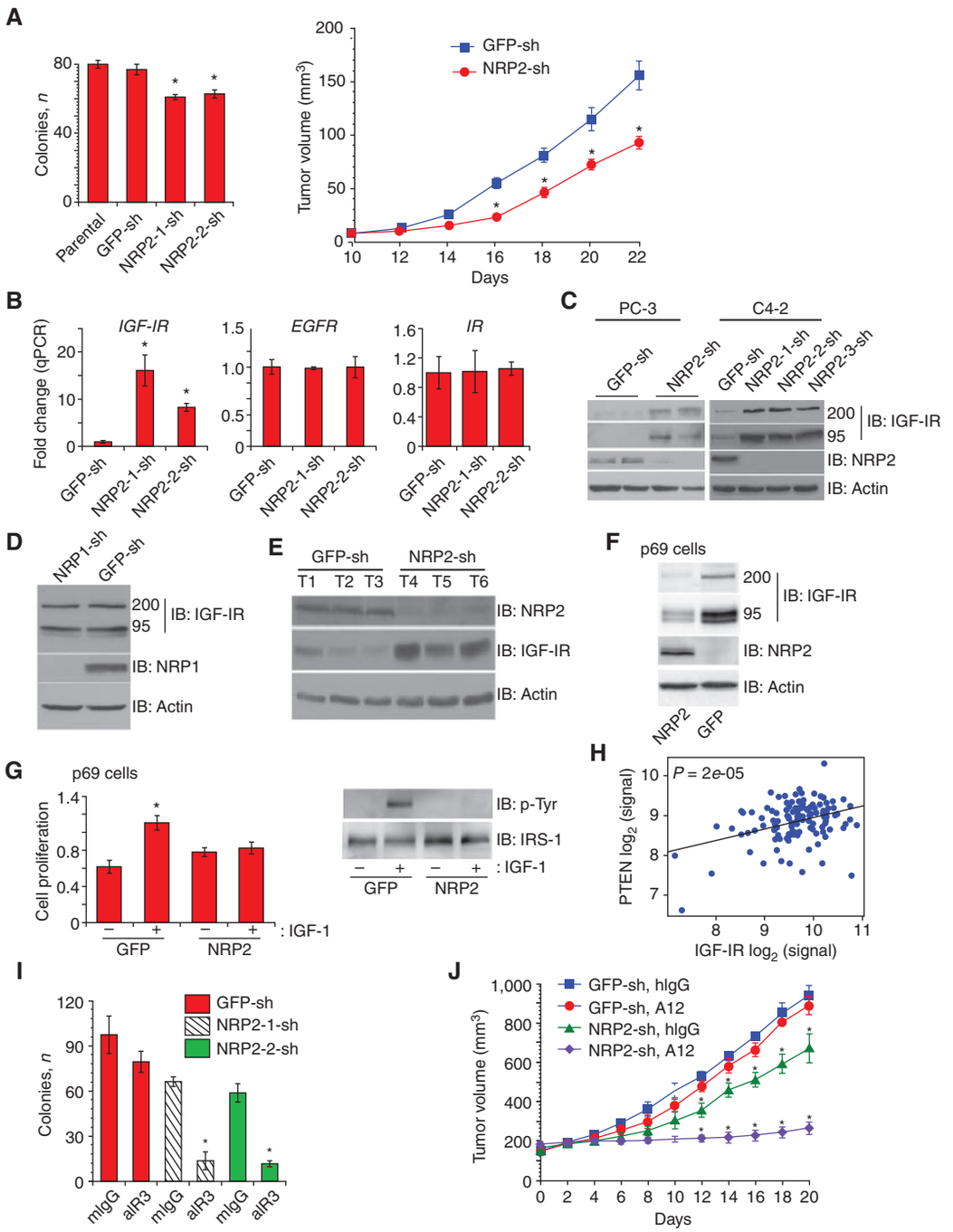

Figure 4. NRP2 represses IGF-IR expression and signaling. **A,** PC3 cells were either not transfected (parental) or transfected with either a GFP-shRNA (GFP-sh) or 2 independent NRP2 shRNAs (NRP2-1-sh and NRP2-2-sh) and analyzed for growth in soft agar (left graph). These transfectants (GFP-sh or NRP2-sh) were implanted in immunocompromised mice, and xenograft growth was measured on alternate days ($n = 8$; right graph). **B,** expression of *IGF-IR*, *EGFR*, and *insulin receptor (IR)* mRNA was quantified in PC3 transfectants (GFP-sh, NRP2-1-sh, and NRP2-2-sh) by quantitative PCR (qPCR). **C,** extracts from PC3 (GFP-sh or NRP2-sh) and C4-2 (GFP-sh or NRP2-sh) transfectants were immunoblotted (IB) for IGF-IR, NRP2, and actin. **D,** extracts from PC3 transfectants (GFP-sh or NRP1-sh) were immunoblotted for IGF-IR, NRP1, and actin. **E,** extracts from PC3 xenograft tumors (GFP-sh or NRP2-sh) were immunoblotted for NRP2, IGF-IR, and actin. **F** and **G,** prostate epithelial cells (p69) were transfected with either NRP2 or GFP, and extracts were immunoblotted for IGF-IR, NRP2, and actin (**F**). These transfectants were also analyzed for cell proliferation in response to IGF-1 using the MTT assay (middle graph). They were also serum-deprived for 12 hours and stimulated with IGF-1 (50 ng/mL) for 10 minutes. Cell extracts were used to immunoprecipitate IRS-1 and immunoblotted for phosphotyrosine (p-Tyr) or IRS-1 (right blot). **H,** a significant correlation ($P = 2 \times 10^{-5}$) in expression of PTEN and IGF-IR was observed in human prostate specimens ($n = 128$) as described in Results. **I,** PC3 transfectants (GFP-sh, NRP2-1-sh, and NRP2-2-sh) were analyzed for anchorage-independent growth in the presence of either hIgG or an IGF-IR antibody (αIR3, 1 mg/mL). **J,** PC3 transfectants (GFP-sh or NRP2-sh) were implanted in immunocompromised mice. Mice were injected i.p. with either A12 (IGF-IR antibody) or IgG (40 mg/kg, 3 times a week), once the tumor volume reached approximately 150 mm^3. Xenograft growth was measured on alternate days. Two groups of mice (NRP2-sh^+ hIgG and NRP2-sh^+ A12) exhibited significant inhibition of tumor growth compared with the remaining 2 groups (GFP-sh, hIgG and GFP-sh, A12). *, $P < 0.05$. Eight tumors were analyzed for each group.

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levels (Fig. 4F). These cells also express endogenous VEGF (Supplementary Fig. S5D). Consistent with reduced IGF-IR expression, NRP2 expression inhibited IGF-1–stimulated cell proliferation (Fig. 4G, left) and insulin receptor substrate 1 (IRS-1) phosphorylation (a substrate of IGF-IR tyrosine kinase; Fig. 4G, right). On the basis of these results, it is expected that PTEN-positive tumors, which express low levels of NRP2, have high IGF-IR levels. This hypothesis was confirmed by analyzing gene expression derived from human prostate cancer specimens ($n = 128$; ref. 34). We found a positive correlation between IGF-IR and PTEN expression ($P = 2 \times 10^{-5}$; Fig. 4H).

The compensatory relationship between NRP2 and IGF-IR expression suggested that inhibition of both receptors would be more effective at impeding tumor growth than inhibition of either receptor individually. To test this hypothesis, we investigated the effect of an IGF-IR inhibitory antibody on the ability of control PC3 cells or cells in which NRP2 expression had been ablated to grow in soft agar or in immunocompromised mice. A significant effect of combined NRP2 downregulation and IGF-IR inhibition was evident in both soft agar growth *in vitro* (Fig. 4I) and tumor growth *in vivo* (Fig. 4J; $P < 0.01$). Indeed, combined treatment resulted in complete inhibition of tumor growth (Fig. 4J). The inhibition of NRP2 and IGF-IR did not change the expression of the insulin receptor (Supplementary Fig. S6A), discounting the possibility of compensation by this receptor. Moreover, combined treatment significantly increased the cleavage of caspase-3, suggesting activation of cell death (Supplementary Fig. S6B). Addition of A12 (the clone name of the IGF-IR inhibitory antibody) blocked IGF-1–stimulated tyrosine phosphorylation of IRS-1, validating the functionality of this antibody (Supplementary Fig. S6C). Further evidence in support of our findings was provided by the effect of A12 on PTEN-null (PC3) and PTEN-postitive (VCaP) prostate cancer cell lines. In agreement with our hypothesis, A12 inhibited cell proliferation of VCaP but not PC3 cells (Supplementary Fig. S6D).

VEGF/NRP2 Signaling Regulates Bmi-1, Which Represses *IGF-IR* Transcription

To establish the mechanism by which NRP2 represses the IGF-IR, we focused on our novel finding that NRP2 is required to maintain expression of Bmi-1, a Polycomb group transcriptional repressor that has been implicated in PTEN deletion–induced prostate cancer (16). Specifically, NRP2 loss in both PC3 and C4-2 cells diminished Bmi-1 protein expression markedly (Fig. 5A), as well as *Bmi-1* mRNA expression in PC3 cells (Fig. 5B, left). This effect of NRP2 loss on Bmi-1 expression was also observed in PC3 xenografts (Fig. 5B, right). These data linking NRP2 to Bmi-1 further imply that NRP2 has a role in tumor formation. This effect was manifested in soft agar assays because overexpression of Bmi-1 rescued the impairment in soft agar growth caused by NRP2 downregulation (Fig. 5C). Moreover, analysis of the gene expression data from Taylor and colleagues (34) revealed a positive correlation between expression of Bmi-1 and c-Jun (Fig. 5D; $P = 1 \times 10^{-6}$).

Given that Bmi-1 is a transcriptional repressor and our finding that NRP2 represses expression of the IGF-IR, we examined the ability of Bmi-1 to repress *IGF-IR* transcription. Initially, we observed that depletion of Bmi-1 expression in PC3 cells using shRNAs increased *IGF-IR* mRNA levels (Fig. 5E). Loss of Bmi-1 also increased expression of the IGF-IR protein significantly, confirming the importance of Bmi-1 in repressing IGF-IR expression (Fig. 5F). To investigate the regulation of IGF-IR by Bmi-1, we carried out ChIP using a Bmi-1 antibody and discovered that Bmi-1 binds directly to the *IGF-IR* promoter (Fig. 5G). More specifically, Bmi-1 appears to bind to a region between −74 and −756 in the promoter (Fig. 5G). As further proof that NRP2 represses IGF-IR expression by a Bmi-1–dependent mechanism, we infected PC3 cells that had been depleted of NRP2 with a lentivirus-expressing Bmi-1 and assessed IGF-IR expression. We observed that Bmi-1 represses IGF-IR expression in the absence of NRP2 (Fig. 5H).

Recently, we reported that VEGF/NRP2 signaling activates focal adhesion kinase (FAK) in concert with the α6β1 integrin (35, 36), and we observed that NRP2 and α6β1 interact in prostate carcinoma cells (Supplementary Fig. S6E). To investigate whether NRP2-mediated Bmi-1 expression involves FAK signaling, we evaluated FAK activation in tumor samples *in vivo*. Tumor xenografts from NRP2-depleted cells showed reduced FAK activation (Fig. 6A), and treatment of PC3 cells with a FAK inhibitor reduced expression of Bmi-1 (Fig. 6B). In addition, we also assessed FAK activation in tumor samples by immunostaining. We found that $NRP2^{high}$ ($PTEN^{low}$) tumors express high levels of activated FAK at the membrane compared with $NRP2^{low}$ (PTEN-positive) tumors (Fig. 6C). These data were confirmed by overexpression of constitutively active FAK (CA-FAK; K38A) in NRP2-depleted cells, which resulted in increased expression of Bmi-1 (Fig. 6D). To analyze the effect of c-Jun expression on FAK activation, we used LNCaP cells expressing low levels of NRP2 and observed that c-Jun expression increases NRP2, as well as FAK activation (Fig. 6E). Similarly, downregulation of c-Jun reduced FAK activation and Bmi-1 expression, and led to increased IGF-IR levels (Fig. 6F).

A critical issue is whether the observed effects of NRP2 on Bmi-1 and IGF-IR expression involve VEGF, an NRP2 ligand. To address this issue, we downregulated VEGF expression in PC3 cells using either siRNA or shRNA and observed that decreased VEGF expression is associated with decreased Bmi-1 and increased IGF-IR expression (Fig. 7A and B; Supplementary Fig. S6F). Moreover, exogenous VEGF increased Bmi-1 expression in a dose-dependent manner (Supplementary Fig. S6G). Our data also imply that VEGF expression should be higher in prostate tumors with PTEN loss than in those with PTEN-positive tumors and that it should positively correlate with NRP2 and inversely correlate with IGF-IR expression. To assess this hypothesis, quantitative PCR was carried out using RNA isolated from 12 prostate tumors (6 $PTEN^{low}$ and 6 $PTEN^{high}$). The $PTEN^{low}$ tumors exhibited relatively high expression of NRP2 and VEGF but low expression of the IGF-IR compared to the $PTEN^{high}$ tumors (Fig. 7C). The expression pattern of VEGF is consistent with our finding that VEGF expression in tumor cells correlates with Gleason grade (20). To exclude the possibility that VEGF regulation of Bmi-1 is mediated by other VEGF receptors, especially VEGF tyrosine kinase

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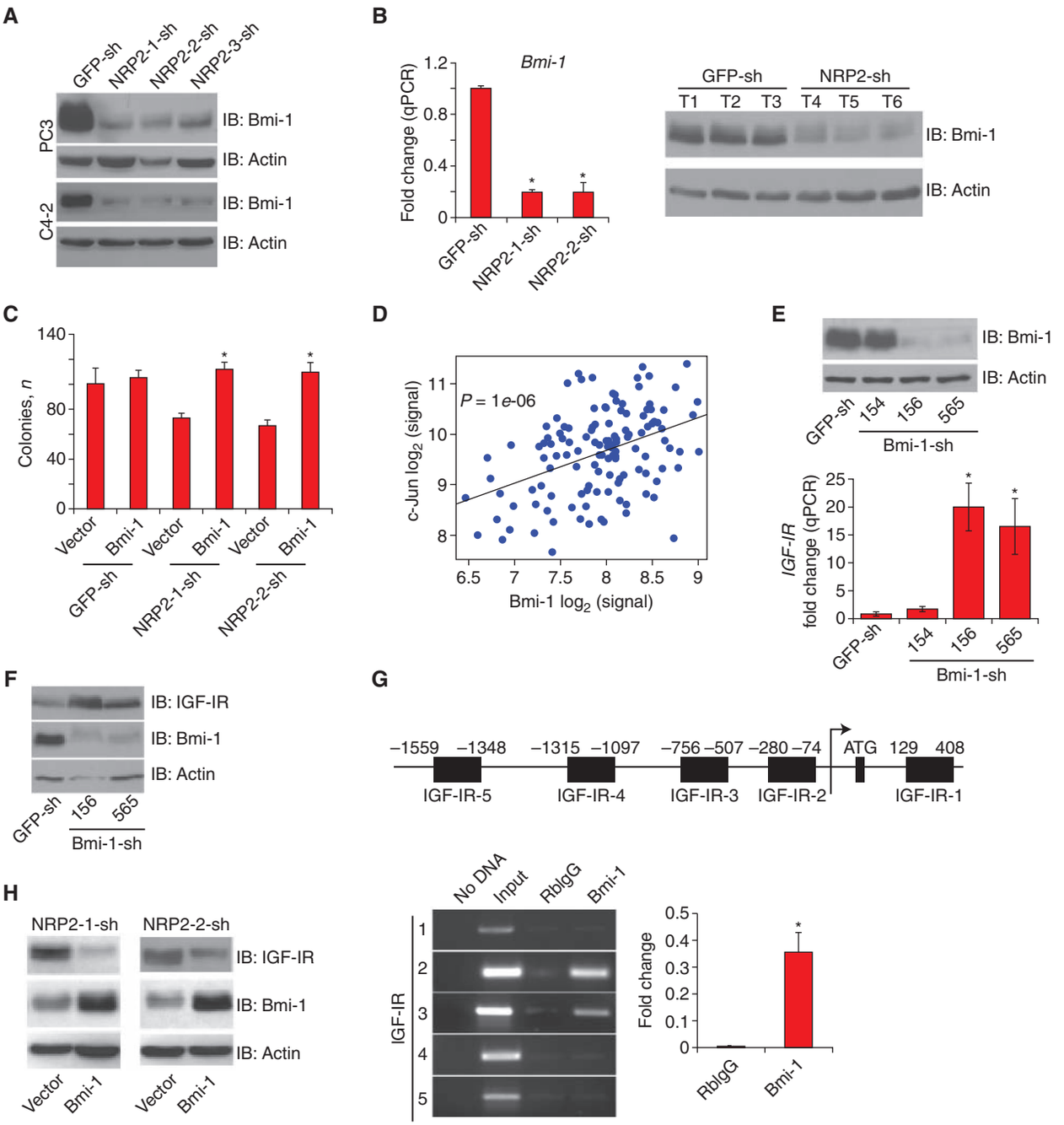

Figure 5. NRP2 sustains expression of Bmi-1, which represses *IGF-IR* transcription. **A** and **B,** Bmi-1 expression was evaluated in PC3 and C4-2 transfectants (GFP-sh, NRP2-1-sh, NRP2-2-sh, and NRP2-3-sh) by immunoblotting (IB; A) and quantitative PCR (qPCR; **B,** left graph). Extracts from PC3 xenograft tumors (GFP-sh or NRP2-sh) were immunoblotted for Bmi-1 and actin (B, right blot). **C,** PC3 transfectants (GFP-sh, NRP2-1-sh, and NRP2-2-sh) were infected with lentivirus particles expressing either GFP or Bmi-1 and analyzed for growth in soft agar. Expression of Bmi-1 increases anchorage-independent growth. *, $P < 0.05$. **D,** a significant correlation ($P = 1 \times 10^{-6}$) in the expression of c-Jun and Bmi-1 was observed in human prostate specimens ($n = 128$) as described in Results. **E,** PC3 transfectants (GFP-sh or Bmi-1-sh) cells were immunoblotted for Bmi-1 and actin (top blot). PC3 transfectants (GFP-sh, Bmi-154-sh, Bmi-156-sh, and Bmi-565-sh) were analyzed for *IGF-IR* mRNA by qPCR. Note: Bmi-1-154-sh did not decrease Bmi-1 expression and showed no effect on *IGF-IR* mRNA (bottom graph). *, $P < 0.05$. **F,** PC3 transfectants (GFP-sh, Bmi-156-sh, and Bmi-565-sh) were immunoblotted for Bmi-1, IGF-IR, and actin. **G,** ChIP analysis of Bmi-1 binding to the *IGF-IR* promoter. A schematic of the *IGF-IR* promoter with highlighted boxes (IGF-IR-1-5) representing the primers used to amplify the ChIP DNA by semiquantitative PCR. The arrow indicates the transcriptional start site and ATG indicates the translation start codon. ChIP was conducted using a Bmi-1 antibody or rabbit IgG and precipitated DNA was amplified by PCR using primers specific for regions 1 to 5. Bmi-1 binding to the *IGF-IR* promoter was confirmed by qPCR. **H,** PC3 transfectants (NRP2-1-sh and NRP2-2-sh) were infected with lentivirus particles expressing either GFP or Bmi-1, and extracts were immunoblotted for IGF-IR, Bmi-1, and actin. *, $P < 0.05$.

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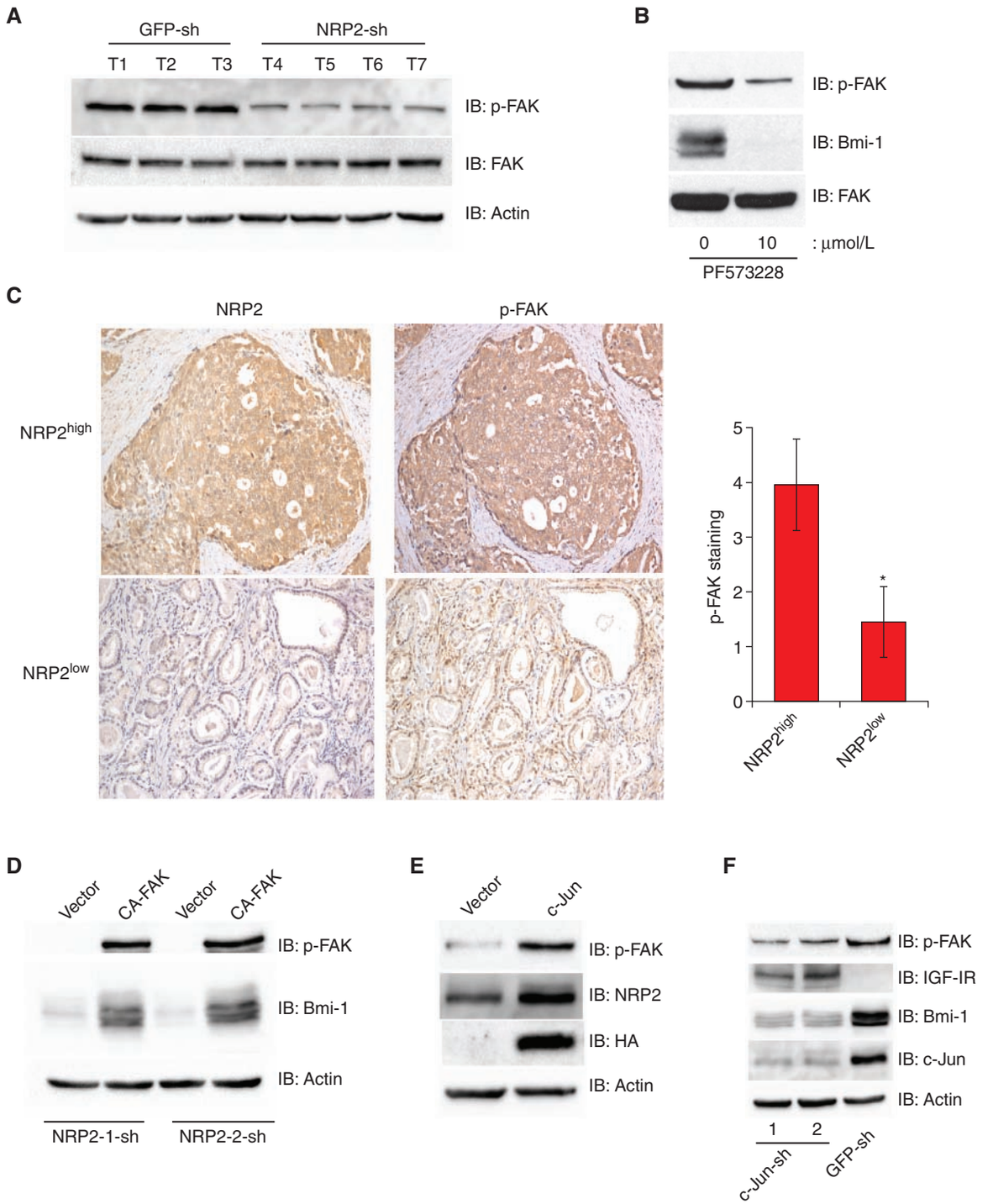

Figure 6. FAK mediates NRP2-stimulated Bmi-1 expression. **A,** extracts from PC3 xenograft tumors (GFP-sh or NRP2-sh) were immunoblotted (IB) for phospho-FAK [p-FAK (pY397)], total FAK, and actin. **B,** PC3 cells were incubated with a FAK inhibitor PF573228 (10 μmol/L), and extracts were immunoblotted (IB) for p-FAK, Bmi-1, and FAK. **C,** human prostate cancer specimens ($n = 24$) were immunostained for NRP2 and p-FAK (pY397). NRP2 staining was scored as either low (score 0 or 1) or high (score 2–5). p-FAK staining was scored at a scale of 1 to 5. The graph summarizes the intensity of p-FAK in $NRP2^{low}$ and $NRP2^{high}$ tumors. There is significantly higher p-FAK in the $NRP2^{high}$ tumors than in the $NRP2^{low}$ tumors (*, $P = 0.00004$). **D,** PC3 transfectants (NRP2- 1-sh and NRP2-2-sh) were transfected with either CA-FAK (K38A) or vector alone, and extracts were immunoblotted for p-FAK (pY397), Bmi-1, and actin. **E,** LNCaP cells were transfected with either c-Jun or vector and immunoblotted for p-FAK (pY397), NRP2, HA, and actin. **F,** PC3 cells were transfected with c-Jun-shRNAs (1 and 2) or a GFP-shRNA and immunoblotted for p-FAK (pY397), IGF-IR, c-Jun, Bmi-1, and actin.

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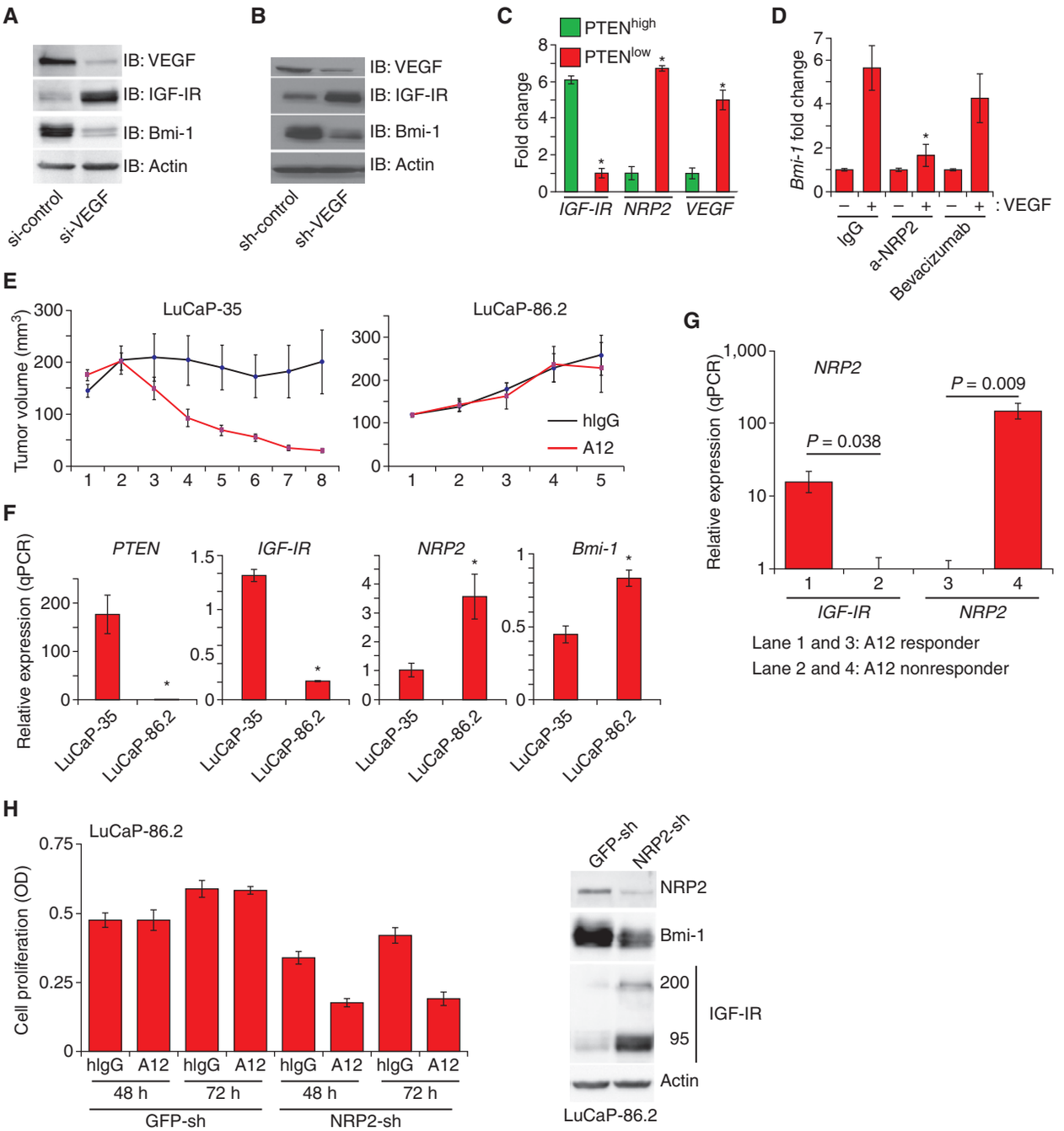

Figure 7. VEGF regulates Bmi-1 and IGF-IR expression. **A** and **B,** PC3 cells were either transfected with VEGF siRNA Smartpool (**A**) or infected with a VEGF-shRNA (**B**). Extracts were immunoblotted (IB) for VEGF, NRP2, Bmi-1, and actin. **C,** microdissected tumor cells from PTEN^{high} ($n = 6$) or PTEN^{low} ($n = 6$) prostate cancer specimens were analyzed for *VEGF, NRP2,* and *IGF-IR* mRNA expression by quantitative PCR (qPCR). PTEN expression correlates positively with IGF-IR and negatively with NRP2 and VEGF expression. **D,** PC3 cells expressing VEGF-sh were incubated with VEGF in the presence of control IgG, an NRP2 antibody or bevacizumab. Cells were analyzed for expression of *Bmi-1* by qPCR. NRP2 expression predicts response to IGF-IR therapy (**E–H**). **E,** graphs display the volume of LuCaP xenograft tumors (±SEM) of 10 castrated mice after A12 or human IgG treatment. **F,** LuCaP xenografts (35 and 86.2) were analyzed for *IGF-IR, NRP2, PTEN,* and *Bmi-1* expression by qPCR. The A12 nonresponder (86.2, PTEN-null) had significantly higher *NRP2* and *Bmi-1* expression than did the A12 responder. *, $P < 0.05$. **G,** *IGF-IR* and *NRP2* expression was quantified by qPCR in 15 A12 responders and 8 nonresponder LuCaP xenografts. Data show 150-fold higher *NRP2* expression in the nonresponders than in responders and only a 10-fold increase in *IGF-IR* expression in the responders compared with the nonresponders. **H,** freshly isolated cells from LuCaP-86.2 tumors were infected with lentivirus expressing either GFP-sh or NRP2-sh. Cell proliferation was measured in the presence of either hIgG or A12 up to 72 hours using MTT assay (left graph), and the expression of NRP2, Bmi-1, and IGF-IR was assessed by immunoblotting (right blot). OD, optical density.

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receptors, we used bevacizumab, which inhibits the interaction of VEGF with these receptors but not with NRP2 (22). An NRP2-blocking antibody inhibited VEGF induction of Bmi-1 expression, but bevacizumab had no significant effect (Fig. 7D).

NRP2 Is a Biomarker that Predicts Response to IGF-IR Therapy

Collectively, the data generated in this study indicate that NRP2 expression in tumor cells is a potential biomarker to predict the efficacy of IGF-IR inhibitors, which are currently in clinical trials. To test this latter possibility, we used 2 LuCaP xenograft models: LuCaP-35 and LuCaP-86.2. LuCaP-35 is sensitive to the IGF-IR inhibitor A12 and tumor growth is substantially inhibited upon A12 treatment. In contrast, LuCaP-86.2 is insensitive to A12 (Fig. 7E). We detected significantly reduced expression of IGF-IR and increased expression of NRP2 and Bmi-1 in LuCaP-86.2 (PTEN-null) compared with LuCaP-35 (PTEN-positive) tumors. Conversely, LuCaP-35 tumors expressed significantly higher levels of IGF-IR than NRP2 and Bmi-1 (Fig. 7F). These data were further strengthened by the analysis of an additional 15 A12 responders and 8 A12 nonresponders (Fig. 7G; Supplementary Fig. S7). As shown in Fig. 7G, we detected 150-fold higher NRP2 expression in the nonresponders than in responders and only a 10-fold increase in IGF-IR expression in the responders compared with the nonresponders. To further substantiate our finding that NRP2 loss can enhance sensitivity to IGF-IR inhibition, we ablated NRP2 expression in LuCaP-86.2 cells and observed a significant increase in the response to A12 as assessed by cell proliferation (Fig. 7H). Importantly, this response is associated with a significant decrease in Bmi-1 expression and increase in IGF-IR expression (Fig. 7H).

DISCUSSION

Our data highlight a novel role for VEGF/NRP2 signaling in prostate cancer cells that impacts our understanding of the biologic nature of this disease, especially aggressive prostate cancer, and our approach to therapy. Specifically, we conclude that the VEGF receptor NRP2 is induced by PTEN loss and that VEGF/NRP2 signaling regulates the expression of Bmi-1, a key effector of prostate tumorigenesis induced by PTEN deletion (16). The consequences of this mechanism are significant for several reasons, including our finding that Bmi-1 represses transcription of the *IGF-IR* and abrogates IGF-IR signaling. We exploited these results to show that NRP2 is a novel biomarker for high-grade prostate cancer that can be used to predict response to IGF-IR therapy. Importantly, our work indicates that combined NRP2 and IGF-IR inhibition is an effective approach for impeding tumor growth that overcomes the resistance caused by inhibiting either receptor independently.

A major conclusion from our work is that NRP2 expression and function are linked directly to oncogenic transformation associated with loss of PTEN function. More specifically, we show that PTEN loss induces NRP2 expression by activating JNK1/c-Jun and that an inverse correlation between PTEN and NRP2 expression is evident in both the $Pten^{pc-/-}$ mice and in human prostate cancer. These expression data were substantiated by our finding that VEGF/NRP2 signaling regulates the expression of Bmi-1, a Polycomb group transcriptional repressor implicated in prostate tumorigenesis (16). Although a previous study had foreshadowed a role for NRP2 in the xenograft growth of colorectal carcinoma cells (37), our study is the first to show that an oncogenic pathway, that is, c-Jun activation induced by PTEN loss, regulates NRP2. Moreover, our finding that NRP2 expression increases with Gleason grade is consistent with our previous finding that VEGF expression in cancer cells also increases with Gleason grade (20), supporting the hypothesis that autocrine VEGF/NRP2 signaling is characteristic of high-grade, aggressive prostate cancer. We do not exclude the possibility that this signaling pathway involves other VEGF receptors in addition to NRP2. Specifically, prostate carcinoma cells express VEGFR2 but lack expression of VEGFR1 (Supplementary Fig. S2E). The absence of VEGFR1 is consistent with previous reports and may result from hypermethylation of the *FLT1* gene encoding VEGFR1 in prostate cancer (23, 24). Nonetheless, the possibility that VEGFR2 contributes to NRP2 signaling is mitigated but not discounted by our finding that bevacizumab, which inhibits the interaction of VEGF with tyrosine kinase VEGF receptors but not with NRP2 (22), did not inhibit the ability of VEGF to regulate Bmi-1.

Unexpectedly, we identified NRP2 as a novel c-Jun target that is regulated by PTEN. The implication of these findings is that VEGF/NRP2 signaling is a component of the mechanism by which PTEN loss induces prostate cancer. Our key findings that VEGF/NRP2 signaling regulates Bmi-1 expression and that there is a strong correlation between NRP2 and Bmi-1 in human prostate cancer buttress this conclusion. The significance of these data is that they provide a causal link between VEGF/NRP2 signaling and a mechanism of prostate tumorigenesis and they build upon the seminal report that Bmi-1 regulates the self-renewal of prostate stem cells and malignant transformation (16). Our data provide a specific mechanism (autocrine VEGF/NRP2 signaling) that contributes to Bmi-1 regulation in prostate cancer. Of note, this mechanism is consistent with the hypothesis that tumor stem cells secrete growth factors in an autocrine manner to protect themselves from differentiation and to support their self-renewal (38).

An important issue that arises from our data is how VEGF signals through NRP2, given that NRP2 lacks intrinsic signaling function. We hypothesize that the specific interaction between NRP2 and the α6β1 integrin (CD49f) potentiates this signaling. This hypothesis is based on our previous finding that NRP2 facilitates the α6β1-mediated activation of FAK and Src (36) and our observation here that NRP2 and α6β1 interact in prostate carcinoma cells. Interestingly, the association of NRP2 with α6β1 does not require the cytoplasmic domain of NRP2 (Supplementary Fig. S6E), indicating that this association occurs in the membrane, perhaps in microdomains enriched for these receptors. We stress that our findings do not discount a role for tyrosine kinase VEGF receptors in promoting NRP2 signaling. Numerous studies have shown that the neuropilins can enhance the function of these receptors (6, 7). It is important to note, however, that even if such VEGF receptors contribute to the signaling pathway we describe, NRP2 is essential and a prime target for therapeutic intervention.

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Moreover, the data presented in Fig. 4J indicate that targeting NRP2 and IGF-IR is sufficient to block tumor growth in the absence of any other VEGF receptor inhibition.

We implicate FAK as a downstream effector of VEGF/NRP2 signaling that regulates Bmi-1 expression. This ability of FAK to regulate Bmi-1 is novel and it adds to our understanding of how FAK contributes to tumorigenesis. FAK expression is increased in prostate cancer cells compared with prostatic epithelium, and there is evidence that FAK participates in prostate tumorigenesis (39). FAK is also necessary for mammary tumorigenesis and for the function of mammary tumor stem cells (40), but the mechanism by which FAK functions in this capacity has not been elucidated. Clearly, the ability of FAK to regulate Bmi-1 provides one such mechanism. As mentioned above, it is likely that the α6β1 integrin is a component of this mechanism (35, 36).

Our data support the conclusion that IGF-IR expression and signaling are diminished in high-grade, aggressive prostate cancer, most likely as a result of PTEN loss. Although this conclusion is unexpected because IGF-IR signaling is thought to promote tumor proliferation in prostate and other cancers (41), this receptor can also promote differentiation (42, 43) and this function might need to be impeded by VEGF/NRP2 signaling for the genesis of high-grade, aggressive cancer. This possibility is supported by the report that abrogation of functional IGF-IR expression actually promoted earlier emergence of more aggressive, less-differentiated carcinomas in a p53-compromised model of prostate cancer (44).

The data presented have significant implications for prostate cancer therapy, especially because the clinical use of IGF-IR inhibitors is feasible (17) and NRP2 inhibitors are available (13). On the basis of our data, most of the ongoing IGF-IR clinical trials will not succeed because these trials recruited many patients with high-grade, aggressive prostate cancer, which expresses NRP2 but lacks IGF-IR and PTEN expression. Inhibition of the IGF-IR may reduce the burden of low-grade tumors, but such tumors likely contain a small population of NRP2-positive cells that are resistant to IGF-IR inhibition and will cause relapse unless this VEGF receptor is also targeted. NRP2 inhibition could be a very effective therapy for aggressive, high-grade tumors provided that the IGF-IR is also inhibited to reduce tumor burden caused by compensatory signaling. Furthermore, this study identifies NRP2 as a novel biomarker for predicting response to IGF-IR therapy. A critical finding in this context is that NRP2 is a much more robust biomarker than the IGF-IR itself. This finding is significant because the IGF-IR is also expressed in normal glands, in contrast with NRP2. Importantly, the data presented provide a rationale for initiating clinical trials that combine inhibitors of both NRP2 and IGF-IR.

METHODS

Clinical Specimens

Tissue samples of defined Gleason grade, lymph node, and bone metastases were obtained from the University of Massachusetts Medical School (UMASS) Cancer Center Tissue Bank with approval of the Institutional Review Board (IRB) of UMASS Medical School (Worcester, MA). The IRB granted a waiver for obtaining patients' consent in accordance with NIH guidelines because these are pre-existing, de-identified specimens. Formalin-fixed, paraffin-embedded sections either from primary tumors, lymph node metastases, or bone metastases were stained using either NRP2 or control IgG antibody as described before (20). Frozen sections were microdissected by laser capture microscopy (Arcturus PixCell 2) as described elsewhere (20) to obtain pure populations of tumor cells of defined Gleason grades. RNA was isolated from these microdissected samples using the RNeasy Kit (Qiagen) and prepared cDNA using Superscript II reverse transcriptase (Invitrogen). Quantitative PCR was carried out using the TaqMan Assay Kit (Applied Biosystems): PTEN (Hs02621230_s1), NRP2 (Hs00187290_m1), VEGF (Hs00900055_m1), IGF-IR (Hs00609566_m1), and GAPDH (Hs9999905_m1). Tissue microarrays containing 52 primary prostate cancer tissues were also used.

Reagents and Antibodies

Bmi-1 (Cell Signaling), actin (Sigma), IGF-IR (Cell signaling), NRP2 (C-9, H-300, Santa Cruz and R&D), p-FAK (Y397, Invitrogen) a-VEGF (Calbiochem), insulin receptor (Santa Cruz), cleaved caspase-3, PTEN (Cell Signaling), p-Tyr (PY99), NRP1 (Santa Cruz), p-AKT (Ser473), pS6K, pFOXO1 (Ser256), FOXO1 (L27), AKT, S6K, p-c-Jun (S73, Cell Signaling), c-Jun (Santa Cruz), IRS-1 (Bethyl), and CD49f (AA6A; kindly provided by Dr. A. Cress, University of Arizona Cancer Center, Tucson, AZ). Bevacizumab was obtained from the pharmacy of UMASS Memorial Medical Center. A12, cixutumumab, was kindly supplied by Dr. Dale Ludwig from ImClone Systems Incorporated. Immunohistochemistry for p-AKT was conducted using D9E antibody (Cell Signaling). IGF-IR antibody α-IR3 was purchased from Abcam and PF573228 (FAK inhibitor) from Tocris.

Constructs

The PTEN plasmid was provided by Dr. Alonzo Ross (UMASS Medical School); Bmi-1-ires-eGFP cloned into FUGW was obtained from Addgene; c-Jun expression constructs (HA-tagged) were provided by Dr. Dirk Bohmann (University of Rochester Medical Center, Rochester, MN); CA-FAK (K38A) construct was provided by Jun-Lin Guan (University of Michigan, Ann Arbor, MI). Lentiviruses vectors expressing NRP2 shRNAs (Open Biosystems; clone ID TRCN0000063308, TRCN0000063309 or TRCN0000063312), VEGF shRNA (TRCN0000003343), Bmi-1 shRNA (TRCN0000020154, TRCN0000020156, TRCN0000012565), or c-Jun shRNAs (provided by S. Huang, Georgia Health Sciences University, Augusta, GA) or a GFP control (Open Biosystems, RHS4459) were used.

Cells and Transfectants

Prostate cancer cells, LNCaP, PC3, VCaP, DU145 (American Type Culture Collection), C4-2 (UroCor, Inc.), or normal prostate immortalized cell lines RWPE-1 (provided by Dr. SK Batra, University of Nebraska Medical Center, Omaha, NE), PNT1A (provided by Dr. M. Ittmann, Baylor College of Medicine, Houston, TX), and PZ-HPV7 (provided by Dr. T.C. Chen, Boston University, Boston, MA) were used. PC3-M cells (45) were kindly provided by Dr. Paul Mak (UMASS Medical School). These cell lines were not authenticated. Lentiviruses containing NRP2 shRNAs, VEGF shRNA, Bmi-1 shRNA, or c-Jun shRNAs or a GFP control were generated, titrated according to the manufacturer's instructions, and used to infect cells following standard protocols. Stable cell transfectants were generated by puromycin selection (2 mg/mL). In some experiments, cells were transfected with VEGF siRNA (Smartpool, Dharmacon) or scrambled control siRNA.

Anchorage-Independent Growth Assays

Growth in soft agar was assayed by scoring the number of colonies formed in medium [Dulbecco's Modified Eagle's Media (DMEM)

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supplemented with 10% FBS] containing 0.3% agarose, with a 0.5% agarose medium underlay as described (46). PC3-parental, PC3-GFP-sh, or PC3-NRP2-sh (NRP2-sh-1 or NRP2-sh-2) cells were seeded on 60-mm diameter plates in triplicate. Cells were fed with 1.5 mL of suspension medium (DMEM supplemented with 10% FBS) every 3 days. The number of colonies larger than 100 μm was counted after 14 days.

Pten$^{pc-/-}$ Mice

The *Pten*$^{pc-/-}$ mice (26) was backcrossed more than 10 times to obtain a BALB/c background. Specimens of PIN (6 weeks) or invasive prostate tumors (20 weeks) from these mice and mice with wild-type *Pten* were stained for NRP2 or NRP1 expression using immunohistochemistry. All animal experiments were in accordance with institutional guidelines and were approved by the Institutional Animal Care and Use Committee.

Luciferase Assays

Cells were transfected with the luciferase construct and *Renilla* luciferase construct to normalize for transfection efficiency. Relative light units were calculated as the ratio of firefly luciferase to *Renilla* luciferase activity (normalized luciferase activity). The protocol used for transfection and measurement of luciferase activity has been described previously (20). We used an NRP2 promoter luciferase construct (−3,000/+195) or the same construct with AP1 mutations. A full-length wild-type c-Jun promoter (−1,780/+731) luciferase construct was provided by Dr. Wayne Vedeckis (LSU Health Sciences Center, New Orleans, LA) and used to study the effect of PTEN expression.

ChIP Assays

ChIP assays were conducted according to our published protocol (47). All ChIP experiments were carried out at least 3 times and the variation was less than 20%. The primers used to amplify the IGF-IR and NRP2 promoters, as well as other genes analyzed in this study, are provided in Supplementary Fig. S8.

Xenografts

Tumor fragments (20–30 mm^3) from the human prostate tumor xenografts (LuCaP-35 or LuCap-86.2; established by co-author R.L. Vessella) were implanted s.c. into 6-week-old–castrated severe combined immunodeficient mice as previously described (48, 49). Once the implanted tumor reached a volume less than 100 mm^3, animals were randomized into 2 groups (10 mice per group). Group 1 animals received human IgG treatment and were designated as controls. Group 2 animals received A12 treatment at a dose of 40 mg/kg. All treatments were given intraperitoneally (i.p.) 3 times a week for 4 weeks. Tumors were measured twice weekly and tumor volume was estimated by the formula: volume = $L \times W^2/2$. Single-cell suspensions of LuCaP-86.2 cells were prepared by resecting the s.c. xenografts and cutting them into small pieces in DMEM/F12 containing 10% FBS. The small pieces were mechanically dissociated, and cell viability was determined by trypan blue counting as described (50). Cells were cultured in DMEM/F12 containing 20% FBS and 5 ng/mL EGF. PC3 transfectants (GFP-sh or NRP2-sh) implanted in immunocompromised mice were injected intraperitoneally with either A12 or IgG, once the tumor volume reached approximately 150 mm^3. Xenograft growth was measured on alternate days. All animal experiments were in accordance with institutional guidelines and were approved by the Institutional Animal Care and Use Committee.

Cell-Based Assays

Cell proliferation was measured using MTT assay. Cell invasion assays were conducted using Matrigel-coated Transwell plates.

Disclosure of Potential Conflict of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: H.L. Goel, S.R. Plymate, A.M. Mercurio
Development of methodology: H.L. Goel, C. Chang, B. Pursell, S.R. Plymate
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H.L. Goel, C. Chang, B. Pursell, S. Lyle, H. Adisetiyo, P. Roy-Burman, P.S. Nelson, R.L. Vessella, R.J. Davis, S.R. Plymate
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H.L. Goel, H.S. Xi, C.-C. Hsieh, I.M. Coleman, A.M. Mercurio
Writing, review, and/or revision of the manuscript: H.L. Goel, S. Lyle, C.-C. Hsieh, P. Roy-Burman, R.L. Vessella, R.J. Davis, S.R. Plymate, A.M. Mercurio
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VEGF targets the tumour cell

Hira Lal Goel and Arthur M. Mercurio

Abstract | The function of vascular endothelial growth factor (VEGF) in cancer is not limited to angiogenesis and vascular permeability. VEGF-mediated signalling occurs in tumour cells, and this signalling contributes to key aspects of tumorigenesis, including the function of cancer stem cells and tumour initiation. In addition to VEGF receptor tyrosine kinases, the neuropilins are crucial for mediating the effects of VEGF on tumour cells, primarily because of their ability to regulate the function and the trafficking of growth factor receptors and integrins. This has important implications for our understanding of tumour biology and for the development of more effective therapeutic approaches.

Integrins
A family of more than 20 heterodimeric cell surface extracellular matrix (ECM) receptors. Integrins connect the ECM to the cytoskeleton and can transmit signalling information bidirectionally.

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Vascular endothelial growth factor (VEGF) was identified and isolated as an endothelial cell-specific mitogen that has the capacity to induce physiological and pathological angiogenesis[1,2]. In a separate context, a factor that promotes vascular hyperpermeability, vascular permeability factor, was isolated and later shown to be identical to VEGF[3,4]. This VEGF is now known as VEGFA and is a member of a larger family of growth factors that also includes VEGFB, VEGFC, VEGFD and placental growth factor (PLGF). These family members differ in their expression pattern, receptor specificity and biological functions[5]. VEGFA, which is often referred to as VEGF, has been studied more than the other members of this family and it has several distinct variants ($VEGF_{121}$, $VEGF_{145}$, $VEGF_{148}$, $VEGF_{165}$, $VEGF_{183}$, $VEGF_{189}$ and $VEGF_{206}$). These variants occur because of alternative splicing, and they also differ in receptor specificity and function[5]. Unsurprisingly, the role of VEGFs in angiogenesis and lymphangiogenesis has dominated the VEGF research field since the initial discovery of VEGFs, and these studies have provided considerable insights into the mechanisms that underlie the complex process of angiogenesis[6]. Importantly, these studies provided the foundation for the development of anti-angiogenic therapies that target VEGF and VEGF receptors[7,8].

It has become apparent that the function of VEGF is not limited to angiogenesis and vascular permeability[9]. VEGF, for example, can affect the function of immune cells that are present in the tumour microenvironment and, consequently, it can affect the host response to tumours (see, for example, REF. 10). In addition, VEGF receptors may regulate the function of fibroblasts in the tumour stroma[11] (BOX 1; FIG. 1). One of the most interesting developments is the discovery that autocrine and paracrine VEGF signalling occur in tumour cells and that this signalling contributes to key aspects of tumorigenesis, especially the function of cancer stem cells, independently of angiogenesis (FIG. 1). Signalling downstream of VEGF in tumour cells is mediated by VEGF receptor tyrosine kinases (RTKs) and neuropilins (NRPs). The NRPs have a major role in this signalling because of their ability to interact with and to affect the function of multiple RTKs and integrins. This Review focuses on VEGF signalling in tumour cells and its implications for tumour biology and therapy.

VEGF receptors on tumour cells

VEGF RTKs and NRPs. The hypothesis that VEGF signalling contributes to the functions of tumour cells implies that tumour cells express specific VEGF receptors that mediate this signalling. The classical VEGF receptors are the RTKs VEGFR1 (also known as FLT1), VEGFR2 (also known as FLK1 and KDR) and VEGFR3 (also known as FLT4)[12]. Although the expression of these receptors was initially thought to be limited to endothelial cells, it is now known that most of these receptors are expressed by many tumour types and that their expression correlates with clinical parameters (TABLE 1). VEGFR2 is the predominant RTK that mediates VEGF signalling in endothelial cells and that drives VEGF-mediated angiogenesis[12]. Interestingly, some tumour cells express VEGFR2 and it has a prime role in mediating VEGF signalling (see, for example, REFS 13,14), but the response of other tumour cells to VEGF seems to be independent of this RTK (see, for example, REFS 15,16), which indicates that VEGF signalling in these cells is mediated by other receptors.

VEGFR1 binds to VEGF with a higher affinity than VEGFR2, but the tyrosine phosphorylation of VEGFR1 in response to VEGF is weaker[5]. This observation, together with the existence of an alternatively spliced

Key points

- Tumour cells express vascular endothelial growth factor (VEGF) receptors and respond to autocrine and paracrine VEGF signals.
- VEGF signalling in tumour cells affects tumour functions independently of angiogenesis.
- VEGF signalling in tumour cells is mediated by VEGF receptor tyrosine kinases (RTKs) and neuropilins (NRPs).
- NRPs may be at the centre of VEGF signalling because they regulate the function of RTKs and integrins that are crucial for tumour cell function.
- Autocrine VEGF signalling may be essential for tumour initiation because it regulates the size of the cancer stem cell pool and the self-renewal of cancer stem cells.
- Therapeutic approaches that aim to target NRPs and VEGF RTKs on tumour cells could be useful to promote tumour regression and to diminish the probability of tumour recurrence, especially when used in combination with VEGF-specific antibodies and other modes of therapy.

Plexins
A large family of transmembrane proteins that share homology in their extracellular domains with the MET receptor and semaphorins.

soluble form of VEGFR1, indicates that this RTK can function as a decoy receptor by sequestering VEGF from VEGFR2, thus regulating VEGFR2 signalling[17]. Nonetheless, some tumour cells express VEGFR1 in the absence of VEGFR2 and seem to use this RTK as a signalling receptor to mediate key functions[17–19]. However, the signalling mechanism of VEGFR1 remains to be elucidated. In contrast to VEGFR1 and VEGFR2, VEGFR3 does not bind VEGFA, and this RTK primarily functions in lymphangiogenesis as a receptor for VEGFC and VEGFD[5,17].

Given that some tumour cells seem to lack expression of one or more of the VEGF RTKs but respond to autocrine and paracrine VEGF signals, it can be inferred that other types of receptors mediate or contribute to VEGF signalling in these cells. In this context, during recent years, the NRPs have garnered the most attention as VEGF receptors that function in tumour initiation and progression[20,21]. The NRPs were initially identified as neuronal receptors for class 3 semaphorins, which are axon guidance factors that function in the developing nervous system[22,23]. NRPs primarily function as co-receptors because they lack an intrinsic signalling capability; for example, NRPs form a complex with specific plexins in neurons and other cell types to form functional semaphorin receptors[24,25]. The two NRPs that are expressed in vertebrates (NRP1 and NRP2) are transmembrane glycoproteins that show 44% homology at the amino acid level. They contain four distinct extracellular domains that mediate ligand binding and a short cytoplasmic domain that lacks catalytic activity[21,26,27]. Alternative splicing of NRP1 and NRP2 can produce multiple isoforms, including secreted, soluble forms and NRP2 variants that have differences in their cytoplasmic domains[28]. There is also evidence that NRPs are modified by *O*-linked glycosylation and that this glycosylation can increase ligand binding and receptor expression[11,29–31].

The crucial finding in the context of this Review is that NRPs can function as VEGF receptors and that they are expressed on tumour cells[32]. This seminal finding led to studies aiming to understand the contribution of NRPs to tumour biology. NRPs that are in a complex with specific plexins can also contribute to tumour cell function by functioning as semaphorin receptors[33] (BOX 2). Although there is some indication that plexins contribute to VEGF signalling[34], more data are needed, especially in tumour cells. The NRPs form complexes with VEGF RTKs (VEGFR1 and VEGFR2) and increase the affinity of these receptors for VEGF[35]. The NRPs can also affect the activity of many other receptors that are crucial for tumour cell function, and there is evidence that they may signal independently of other receptors. The crucial issue is whether these functions involve VEGF. In addition, the question of whether NRP1 and NRP2 in their capacity as VEGF receptors differ in their ability to affect tumour cells has not been investigated in depth, apart from the few examples that are cited below.

Regulation of VEGF signalling in tumour cells. The majority of studies that have observed VEGF signalling in tumour cells have characterized this signalling as autocrine[14,16,36–43], although paracrine signalling does occur (see, for example, REF. 44). Moreover, the existence of autocrine VEGF signalling in human tumours is supported by the observation that VEGF is expressed in tumour cells, as shown by immunohistochemical data (TABLE 1), as well as by *in situ* hybridization and by analysis of microdissected tumour cells[45,46]. This reliance on autocrine signalling might reflect the importance of VEGF in sustaining the self-sufficiency or autonomy of tumour cells — a consideration that is highly relevant to aggressive cancers and to the biology of cancer stem cells. Indeed, autocrine VEGF signalling is generally characteristic of more aggressive cancers, including poorly differentiated carcinomas[15,16,46]. More fundamentally, poorly differentiated carcinomas show an embryonic gene expression pattern and the activation of key developmental pathways[47]. There are some data that implicate such pathways in the regulation of VEGF and VEGFR expression in tumour cells. Thus, these data provide

Box 1 | **Other functions of VEGF in the tumour microenvironment**

In addition to affecting endothelial and tumour cells, vascular endothelial growth factor (VEGF) influences tumour function by targeting other cell types in the tumour microenvironment. Notably, immune cells can express VEGF receptors, and the functions of these cells can be regulated by VEGF signalling; for example, $CD4^+$ forkhead box protein P3 $(FOXP3)^+$ regulatory T cells, which suppress an antitumour immune response, express neuropilin 1 (NRP1) and are 'guided' into tumours by VEGF, which functions as a chemoattractant[10]. Ablation of NRP1 in this population of T cells increases the activation of $CD8^+$ T cells and there is a concomitant reduction in tumour growth. Macrophages in the hypoxic tumour microenvironment secrete VEGF, which contributes to the many functions of VEGF in tumours[123]. In addition to their many other functions, fibroblasts in the tumour stroma secrete VEGF. These cells express NRP1 and use it to increase fibronectin fibril assembly, which augments tumour growth; however, whether this process involves VEGF is not known[11].

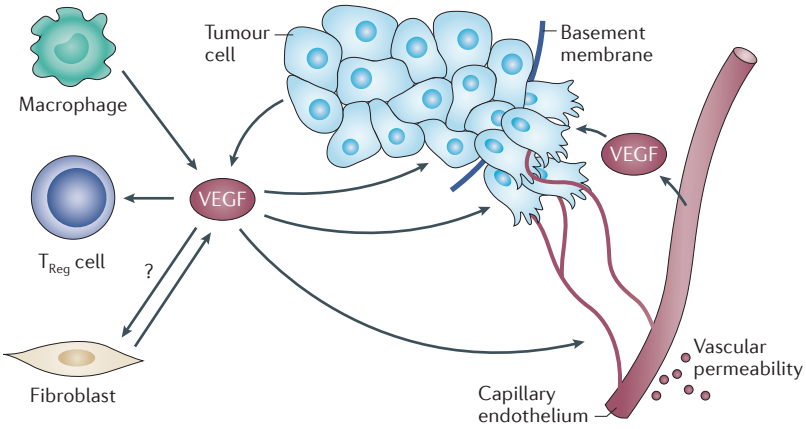

Figure 1 | **VEGF functions in tumours.** Vascular endothelial growth factor (VEGF) that is secreted by tumour and stromal cells, including macrophages, endothelial cells and fibroblasts, has multiple functions in the tumour microenvironment, which involve the ability of VEGF to interact with VEGF receptors that are expressed on different cell types. VEGF functions as a primary stimulus for angiogenesis, which is a process that involves the ability of VEGF receptors to stimulate signalling pathways that induce the proliferation and the migration of endothelial cells, and the ability of these cells to degrade and to remodel the extracellular matrix. These processes culminate in sprouting angiogenesis and the formation of new blood vessels. VEGF can also increase vascular permeability, which results in the deposition of a provisional fibrin matrix that triggers the formation of desmoplastic stroma. By contrast, VEGF secreted by tumour cells functions in an autocrine manner and promotes dedifferentiation and an epithelial–mesenchymal transition phenotype, with a consequent enhancement of tumour invasion and survival, and it can facilitate the function of cancer stem cells (FIG. 3). VEGF can also function as a chemoattractant to recruit regulatory T (T_{Reg}) cells that inhibit an antitumour immune response. Tumour fibroblasts also secrete VEGF. Neuropilin 1 that is expressed on tumour fibroblasts may contribute to tumour growth by nucleating fibronectin fibril formation, but it is not known whether this process involves VEGF. Arrows indicate the source and the targets of VEGF in tumours.

a causal link between tumour dedifferentiation and the activation of autocrine VEGF signalling, but more investigation is needed (see below). This hypothesis is also supported by the finding that expression of VEGF and VEGFR1 is induced by an epithelial–mesenchymal transition (EMT) of colon carcinoma cells[18] — a process that promotes dedifferentiation and progression to more aggressive tumours. Furthermore, VEGF stimulation of normal epithelial cells and differentiated carcinoma cells can induce an EMT[46,48].

However, the mechanism by which the signalling pathways that are associated with oncogenic transformation and dedifferentiation regulate the expression of VEGF and VEGF receptors is still unknown. Given that hypoxia-inducible factor (HIF)-mediated transcription is a major driver of VEGF expression in tumours, it is likely that hypoxia helps to establish autocrine signalling networks in tumour cells. An important observation is that aggressive tumour cells sustain HIF-mediated transcription[49], and mechanisms that have been implicated in inducing VEGF expression, such as RAS transformation[41,50] or EMT[18,46], probably directly affect HIF expression or activation. For example, the loss of oestrogen receptor-β (ERβ) expression that occurs in poorly differentiated prostate cancer and that causes an EMT phenotype, stimulates *VEGFA* transcription in tumour cells by a mechanism that involves transcriptional repression of prolyl hydroxylase 2 (PHD2; also known as EGLN1), which is an enzyme that targets HIF1α for degradation[51].

Several recent studies have provided insights into the mechanism of NRP regulation in cancer. Notably, Hedgehog signalling can induce NRP expression[38,52], which may be part of a positive feedback loop because NRP-mediated VEGF signalling can also induce the expression of the Hedgehog target gene GLI family zinc finger 1 (*GLI1*)[38,53]. The loss of PTEN induces *NRP2* transcription in prostate cancer through a mechanism that involves the JUN N-terminal kinase (JNK)–JUN pathway, which provides a direct link between the loss of a tumour suppressor and the induction of *NRP2* transcription[15]. Interestingly, both JUN and GLI1 can bind to the *NRP2* promoter and may function together to regulate *NRP2* transcription[15,38]. Expression of the transcription factor COUPTF2 (encoded by *NR2F2*) correlates with disease recurrence and progression in prostate cancer, and it can directly stimulate the transcription of *NRP2* (REFS 54,55). COUPTF2 can also suppress Notch signalling[56], which is interesting because there are reports that a Notch ligand — Delta-like 4 (DLL4) — can repress VEGFR2 and NRP1 expression[57], but another ligand (DLL1) can stimulate their expression[58]. Although more work is needed to understand how signalling pathways that contribute to tumour initiation and dedifferentiation regulate the components of VEGF signalling in tumour cells, the fundamental principle of this regulation has been established.

Autocrine VEGF signalling in tumour cells can also be regulated at the level of receptor trafficking, which enables intracellular VEGFR signalling (FIG. 2). Specifically, autocrine NRP1–VEGFR2 signalling in gliomas involves active VEGFR2 that is localized in a cytoplasmic compartment[14]. This finding is indicative of an increasingly popular view that intracellular VEGFR signalling is important[59] and that a key function of NRPs may be to promote the trafficking of VEGFRs and possibly of other growth factor receptors[60]. This mode of regulation has important implications for therapy (see below).

Functional interactions between VEGF receptors and other receptors. An emerging theme in the literature is that VEGF receptors interact with and affect the function of other growth factor receptors, which is a manifestation of growth factor receptor crosstalk (FIG. 2). In addition to the regulation of VEGF RTKs by NRPs, there are numerous reports that VEGF RTKs and NRPs interact with other growth factor receptors. VEGFR2, for example, forms a complex with MET — the receptor for hepatocyte growth factor (HGF) — in response to VEGF stimulation of glioblastoma cells, and VEGFR2 thereby regulates MET signalling[61]. As co-receptors, the NRPs are promiscuous and have numerous interactions with other receptors. NRP1 interacts with the MET receptor and enhances the ability of HGF to stimulate the invasion of pancreatic carcinoma cells[62] as well as the proliferation and survival of gliomas[63]. A direct interaction between the extracellular domain of NRP1 and the epidermal growth factor receptor (EGFR) has

Epithelial–mesenchymal transition
(EMT). A conversion from an epithelial to a mesenchymal phenotype, which is a normal component of embryonic development. In carcinomas, this transformation results in altered cell morphology, the expression of mesenchymal proteins and increased invasiveness.

Hypoxia-inducible factor
(HIF). A dimeric transcription factor that is formed of α- and β-subunits and that is involved in the hypoxia-sensitive regulation of numerous genes, including glycolytic enzymes, glucose transporters and angiogenic factors.

Table 1 | **Expression of VEGFs and VEGF receptors in human cancers***

VEGF or receptor	Cancer
VEGFs	
VEGF	Bladder[129,130], brain[14,131,132], breast‡ (REFS 38,133–135), colon‡ (REFS 87,136), gastric‡ (REF. 137), oral squamous‡ (REFS 138,139), lung‡ (REFS 140–143), mesothelioma‡ (REF. 144), myeloid leukaemia[145], ovarian[146,147], pancreatic[91,148] and prostate§ (REFS 149–151)
VEGFB	Breast‖ (REFS 134,152) and lung[140]
VEGFC	Breast[153], cervical‖ (REFS 153–155), colon‡‖ (REFS 153,156,157), gastric[158], oral squamous[159], lung‡ (REFS 140,153,160) and prostate[153]
VEGFD	Cervical[154], gastric[161] and lung[140]
PLGF	Breast¶ (REF. 162), colon‡ (REF. 136), gastric‡§‖ (REF. 163) and hepatocellular¶ (REF. 164)
VEGF receptors	
VEGFR1	Bladder[130], brain[131,132], breast‡ (REFS 133–135,152,165), colon[18,92], head and neck[166], lung‡ (REFS 140–142), melanoma[167], mesothelioma[144], myeloid leukaemia[145], oesophageal[168], ovarian[86,146,147], pancreatic (REFS 91,148) and prostate (REF. 169)
VEGFR2	Bladder§ (REF. 129), brain[14,131,132,161,170,171], breast‡ (REFS 133,135,172), cervical[173], colon§‖ (REFS 87,174), endometrial‡ (REF. 175), gastric[137], head and neck[166,176], hepatocellular‡ (REF. 177), lung‡ (REFS 140–142,178), melanoma[167], mesothelioma[144], multiple myeloma[179], myeloid leukaemia[145], oesophageal[168], ovarian[86,146,147], pancreatic[91,148], prostate[149,169], renal cell carcinoma[180], squamous[181] and thyroid‖ (REF. 182)
VEGFR3	Breast[153], cervical§ (REFS 153,154), colon‡‖ (REFS 153,156), gastric‡‖ (REFS 158,161), head and neck[159,166], lung‡§ (REFS 140,153,160), oesophageal[168] and prostate[153]
NRP1	Brain‖ (REFS 14,63,183,184), breast‡ (REFS 135,185,186), colon§ (REFS 83,187,188), lung[143,185,189], melanoma[167], ovarian[147,190,191], pancreatic[84,188,192–194] and prostate§¶ (REFS 150,151,195)
NRP2	Bladder[196], breast[38,186,197,198], colon[113,197], lung[143,189,197], melanoma[197,199], ovarian[190], pancreatic[193,194], prostate‡§ (REF. 15) and renal cell§‖ (REF. 200)

NRP, neuropilin; PLGF, placental growth factor; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor. *The data reported are primarily based on immunohistochemical analyses of tumours and indicate expression of VEGFs or VEGF receptors specifically in tumour cells. ‡Studies that showed a correlation between expression and poor survival or outcome. §Studies that showed a correlation between expression and disease stage or progression. ‖Studies that showed a correlation between expression and metastasis. ¶Studies that showed a correlation between expression and recurrence.

been shown, which augments the response of tumour cells to EGF and transforming growth factor-α (TGFα)[64]. This mechanism may contribute to the sustained activation of EGFR that occurs in some advanced cancers. Both NRP1 and NRP2 can interact with TGFβ receptors and can potentiate TGFβ signalling[65,66]. This finding has implications for EMT, which can be induced by either TGFβ signalling or by NRP-mediated VEGF signalling[46,65]. There is also evidence that NRPs can bind to specific growth factors in addition to VEGF and PLGF[67], including HGF[68], basic fibroblast growth factor (bFGF; also known as FGF2)[68], TGFβ[69] and platelet-derived growth factor (PDGF)[70]. However, whether this binding involves VEGF or whether it initiates a signalling response remains to be determined.

VEGF receptors also interact with specific integrins and activate or enhance integrin signalling in tumour cells (FIG. 2). This concept was established using the observation that VEGF signalling that is mediated by VEGFR2 can activate the ligand-binding function of multiple integrins in both endothelial cells and tumour cells by a mechanism that involves the PI3K–AKT pathway[71]. This mode of regulation may be bidirectional because there is also evidence that the αvβ3 integrin can form a complex with VEGFR2 and can increase the level of phosphorylation of this RTK in response to VEGF[72]. NRPs can also associate with specific integrins and can enhance their function in tumour cells[73,74]. A salient example of this interaction occurs between VEGF-bound NRP2 and the α6β1 integrin in breast carcinoma cells. NRP2, but not NRP1, co-immunopurifies with this integrin, and NRP2-mediated VEGF signalling enables α6β1 integrin to bind to its matrix ligand laminin, to engage filamentous actin, to form focal adhesions and to activate focal adhesion kinase (FAK)[74]. Interestingly, NRP2 is localized in focal adhesions that form on laminin, and this observation provides a direct connection between VEGF signalling and focal adhesion dynamics and signalling; this connection is corroborated by data from endothelial cells[75,76].

Collectively, the data that are currently available highlight a crucial role for the NRPs in regulating growth factor receptor and integrin signalling (FIG. 2), and this aspect of NRP function may underlie the contribution of NRPs to tumour biology. However, much remains to be learnt about the mechanisms by which NRPs interact with growth factor receptors and integrins, and how they potentiate their function[77]; for example, does the ability of NRPs to co-immunopurify and to colocalize with RTKs and integrins reflect their association in macromolecular complexes that contain other signalling molecules and endocytic components? Such complexes could regulate and facilitate VEGF signalling and receptor trafficking. The presence of such complexes could be an explanation for most of the data

Focal adhesions
Dynamic, macromolecular protein complexes that link the extracellular matrix to the actin cytoskeleton through integrins.

Box 2 | **Semaphorins and plexins in tumour cells**

A discussion of vascular endothelial growth factor (VEGF) signalling in tumour cells must include a mention of semaphorins — especially class 3 semaphorins (SEMA3s) — because they are secreted by tumour cells, they function as neuropilin (NRP) ligands and they have been implicated in tumour-associated functions. A prevailing idea is that SEMA3s and VEGFs carry out antagonistic effects on tumour cells: SEMA3s inhibit tumour growth, migration and invasion, and, by contrast, the VEGFs have pro-tumorigenic functions[33,124]. This idea is substantiated by the recent report that triple-negative breast tumours, which are poorly differentiated tumours and which have a high frequency of cancer stem cells, are characterized by high VEGFA expression and low SEMA3 expression[125]. However, there are a few reports indicating that SEMA3s can have a pro-tumorigenic function[33,124]. SEMA3s function by binding to NRPs in a complex with plexins — primarily type A plexins. Plexins are the only transmembrane receptors that can directly interact with small GTPases[126]. SEMA3 binding to type A plexins triggers the collapse of the actin cytoskeleton, which results in impaired migration and invasion. Interestingly, other plexin family members such as plexin B1, which binds to SEMA4D, are upregulated or mutated in some cancers, such as prostate carcinomas[127]. In breast cancer, the ability of the receptor tyrosine kinase ERBB2 to activate the pro-invasive small GTPases RHOA and RHOC is mediated by plexin B1, and this effect is independent of NRPs[128]. Clearly, there is much more to be learnt about semaphorins and plexins in cancer, and their relationship to VEGF signalling. An important issue that should be investigated in more detail is the role of plexins in VEGF–NRP signalling.

on VEGF signalling in tumour cells, such as the regulation of RTK activity by NRPs and the role of NRPs in receptor trafficking.

Another timely issue is how NRPs mediate VEGF signalling independently of VEGF RTKs. Several studies have shown VEGF signalling in cells that lack detectable VEGF RTK expression or involvement; for example, NRP-mediated VEGF signalling was reported to promote the initiation of renal cell carcinoma in the absence of detectable VEGF RTK expression[16]. In addition, the ability of NRP2-mediated VEGF signalling to affect prostate cancer is not inhibited by bevacizumab, which blocks VEGF interactions with VEGFRs but not with NRPs[15]. A probable mechanism to explain these phenomena is that NRPs signal by affecting the function of other RTKs and integrins, as discussed above. However, NRPs may signal independently of RTKs (FIG. 2). Specifically, the cytoplasmic domains of NRP1 and NRP2 contain a PDZ-binding domain that can bind to PDZ-containing proteins, especially GIPC1 (also known as NRP-interacting protein (NIP)). GIPC1 is a cytoplasmic scaffolding protein that interacts with a wide range of receptors and that contributes to receptor trafficking and signal transduction[78,79], and it has been implicated in tumorigenesis[79]. A recent study concluded that the NRP1 PDZ-binding domain is required to mediate the PLGF-stimulated growth of medulloblastoma, and that this is independent of VEGFR1 activity[80]. The PDZ-binding domain is thought to function by forming scaffolding complexes that transduce NRP signals; this hypothesis is corroborated by the finding that GIPC1 mediates the interaction of NRP1 with ABL1, which is a tyrosine kinase that could mediate NRP1 signalling[11]. GIPC1 can also function as a 'bridge' to promote the association of receptors that contain PDZ-binding domains such as NRPs and integrins[81]. Interestingly, the NRP2 cytoplasmic domain contains a motif that has a partial consensus sequence to an immunoreceptor tyrosine-based activation motif (ITAM)[31], although there is no evidence yet that this motif is functional.

PDZ-binding domain
(PSD95, DLG and ZO1-binding domain). A structural, protein–protein interaction domain, which is ~80–90 amino acids in length, that often functions as a scaffold for signalling complexes and/or as a cytoskeletal anchor for transmembrane proteins.

Immunoreceptor tyrosine-based activation motif
(ITAM). A motif (YXXL or YXXI) that can be phosphorylated in response to receptor ligation and that functions as a docking site for other proteins involved in signal transduction.

Autophagy
A cellular response in which the cell metabolizes its own contents and organelles to maintain energy production. Although such a process can eventually result in cell death, it can also be used to maintain cell survival in conditions of limiting nutrients.

VEGF-mediated functions in tumour cells

VEGF regulates key functions of established tumour cells. The overarching theme in this Review is that VEGF signalling in tumour cells markedly affects tumour function and that this is independent of VEGF-mediated angiogenesis and vascular permeability. The initial reports that described the effects of VEGF on tumour cells showed that autocrine VEGF signalling — particularly signalling that is mediated by VEGF RTKs and NRPs — can promote the growth, survival, migration and invasion of cancer cells[18,36,62,73,82–91]. Most of these studies implicated dominant signalling pathways (for example, the PI3K–AKT and MAPK pathways) as the mechanism by which VEGF influences these processes; for example, VEGFR1 promotes the migration and the invasion of colorectal carcinoma cells by stimulating the activation of ERK1 or ERK2 as well as the activation of JNK and the consequent translocation of the p65 (also known as RELA) subunit of nuclear factor-κB (NF-κB) into the nucleus[92]. VEGFR1 can also sustain the survival of colorectal carcinoma cells that have undergone an EMT[18]. Several studies have described the ability of NRP-mediated VEGF signalling to affect the survival of tumour cells by activating the PI3K–AKT pathway; for example, NRP1-mediated VEGF signalling is able to sustain the survival of breast carcinoma cells[36,82].

Recent studies have shown that the role of VEGF signalling in tumours might be more complex than initially thought; for example, the above-mentioned VEGF-induced VEGFR2–MET complex in glioblastoma cells contains a tyrosine phosphatase, which inhibits HGF-mediated invasion and mesenchymal transition[61]. These findings need to be reconciled with other reports that implicate VEGF and VEGFR2 in the function of glioma stem cells[14] (see below). In addition, it has been reported that NRP1 on tumour myofibroblasts nucleates fibronectin fibril assembly by a mechanism that involves the α5β1 integrin and that NRP-mediated fibril assembly contributes to tumour growth[11], which improves our understanding of the importance of NRP signalling in the tumour microenvironment. However, this study did not investigate the contribution of specific NRP ligands and it is therefore not known if the mechanism is VEGF dependent. VEGF may also regulate autophagy because it has been shown that NRP2-mediated VEGFC signalling mediated by mTOR complex 1 activates an autophagic mechanism that combats chemotherapy-induced stress, which has implications for the role of VEGF signalling in therapy resistance[93].

VEGF, cancer stem cells and tumour formation. The importance of VEGF and VEGF receptor functions in cancer has been highlighted by the recent reports that autocrine VEGF signalling has a causal role in tumour formation and in the function of cancer stem cells, and these reports have distinguished this growth factor from many others (FIG. 3). Despite some controversy

CD133
A cell-surface glycoprotein, which is also known as Prominin 1, that can be used as a marker for some cancer stem cells.

regarding the existence and the nature of such cells, it is evident that many tumours harbour a small population of cells that have self-renewal potential and the ability to initiate the growth of new tumours[94]. An initial study using a transgenic mouse model showed that autocrine VEGF signalling synergizes with EGFR signalling to promote the development of squamous carcinoma[41]. Importantly, this study concluded that the effect of VEGF is cell autonomous and angiogenesis independent. Mechanistically, VEGF was shown to mediate an autocrine proliferation loop that involves VEGFR1 and NRP1. A rigorous analysis of the early stages of squamous carcinoma formation in the skin resulted in several seminal findings that suggest that autocrine VEGF signalling is directly involved in the function of cancer stem cells[40]. In early-stage tumours or papillomas, cancer stem cells are localized in a perivascular niche that occurs adjacent to endothelial cells. Blocking VEGFR2 reduced the size of the cancer stem cell pool and their self-renewal potential. Conditional deletion of *VEGFA* in the tumour cells of established tumours caused tumour regression by decreasing both microvascular density and the proliferation and renewal of cancer stem cells. Moreover, genetic deletion of *NRP1* prevented the ability of VEGF to promote stem cell-like properties and self-renewal. These findings, which were observed both *in vivo* and *in vitro*, clearly establish the importance of autocrine VEGFA signalling that is mediated by NRP1 and VEGFR2 in cancer stem cells. The localization of cancer stem cells adjacent to endothelial cells infers that tumour cell-derived VEGFA functions both as a paracrine factor to stimulate angiogenesis and as an autocrine factor to regulate cancer stem cells (FIG. 3). However, the localization of cancer stem cells adjacent to the endothelium needs to be reconciled with other reports showing that hypoxia drives the self-renewal of cancer stem cells[95].

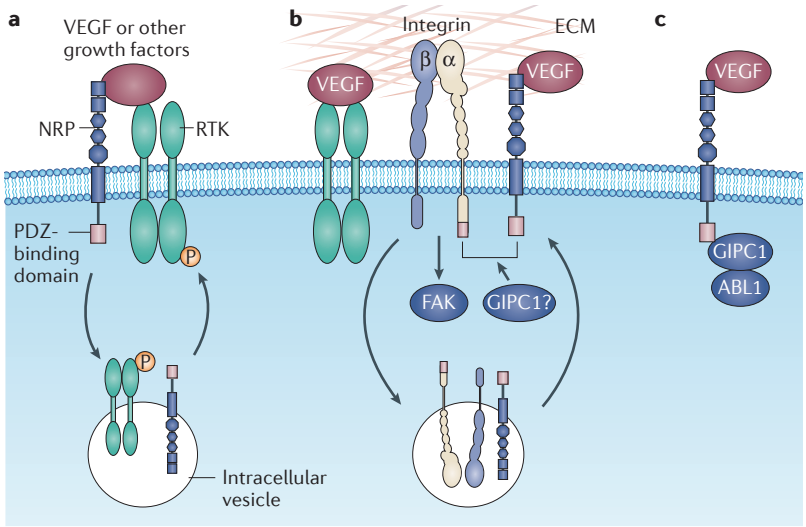

Figure 2 | **Receptor interactions that promote VEGF signalling in tumour cells, and the central role of NRPs.** **a** | Neuropilins (NRPs) interact with and potentiate the signalling function of growth factor receptor tyrosine kinases (RTKs), including vascular endothelial growth factor (VEGF) RTKs. This mode of regulation may be associated with internalization of the RTK and signalling from an intracellular compartment. Several growth factors, including hepatocyte growth factor, basic fibroblast growth factor, platelet-derived growth factor and transforming growth factor-β, directly interact with NRPs, but whether this binding is sufficient by itself to induce a signalling response is not known. **b** | NRPs also interact with specific integrins and activate their ability to bind to extracellular matrix (ECM) ligands, which results in the stimulation of integrin-mediated signalling through focal adhesion kinase (FAK). The RTK VEGF receptor 2 (VEGFR2) can also function in a similar capacity. In addition, NRPs may regulate integrin function by promoting their endocytic recycling. Both NRPs and specific integrin α-subunits (α5 and α6) contain a PDZ (PSD95, DLG and ZO1)-binding domain (Ser-Glu-Ala) at their carboxyl terminus, and PDZ proteins, such as the neuropilin-interacting protein GIPC1 might promote the association of these two classes of receptors. **c** | NRPs may also signal independently of other receptors, possibly by using their PDZ-binding domains to associate with signalling molecules such as ABL1. Note that these proposed mechanisms are not mutually exclusive, and there is the possibility that VEGF signalling in tumour cells involves the formation of macromolecular complexes that integrate components of these mechanisms.

An interesting theme that emerges from the above-described studies and other studies is that distinct VEGF family members and VEGF receptors can be used to facilitate tumour initiation and growth; for example, the two studies of squamous carcinoma formation that are discussed above implicated different VEGF RTKs. An explanation for this may be that the initial study identified a VEGFR1-mediated proliferation loop that contributes to tumour growth[41] and the second study suggested that VEGFR2 is directly involved in the function of cancer stem cells and their self-renewal[40]. It is worth noting that VEGFR1 has yet to be implicated in the function of cancer stem cells or in tumour initiation. Other studies have suggested that the contribution of autocrine VEGF signalling to tumour initiation is independent of VEGF RTKs and is driven by NRP signalling. Such a mechanism has been proposed for renal cell carcinoma[16]. In addition, a PLGF–NRP1 signalling axis that is independent of VEGFR1 contributes to the growth and to the spread of medulloblastomas[80]. Although it is possible that NRP signalling alone mediates autocrine VEGF signalling in this context, a more probable scenario (discussed above) is that NRPs potentiate the function of other non-VEGF receptors that are crucial for the function of cancer stem cells and tumour initiation. This idea is exemplified by our work on the role of VEGF–NRP2 signalling in the initiation of breast cancer. As mentioned above, VEGF–NRP2 signalling can activate the α6β1 integrin[38,74], which is noteworthy because this integrin is necessary for the function of some cancer stem cells[96,97]. Another important aspect of autocrine VEGF signalling in cancer stem cells is that this signalling can occur in an intracellular compartment. VEGFR2 and NRP1 are preferentially expressed on glioma stem cells that are positive for CD133, and ablation of either VEGFR2 or NRP1 in glioma cells *in vivo* increases apoptosis and reduces tumour formation[14]. Importantly, VEGF signalling that is mediated by NRP1 and VEGFR2 maintains a cytosolic pool of active VEGFR2 that may be the source of cell survival signalling and that is resistant to VEGF-specific antibody (bevacizumab) therapy.

Although there are data that clearly implicate VEGF in the function of cancer stem cells and tumour initiation, much less is known about the signalling mechanisms responsible for this function. An example of such a mechanism derives from our work on VEGF–NRP2-mediated activation of the α6β1 integrin. This activation induces activation of the FAK–RAS signalling pathway that culminates in non-canonical Hedgehog signalling,

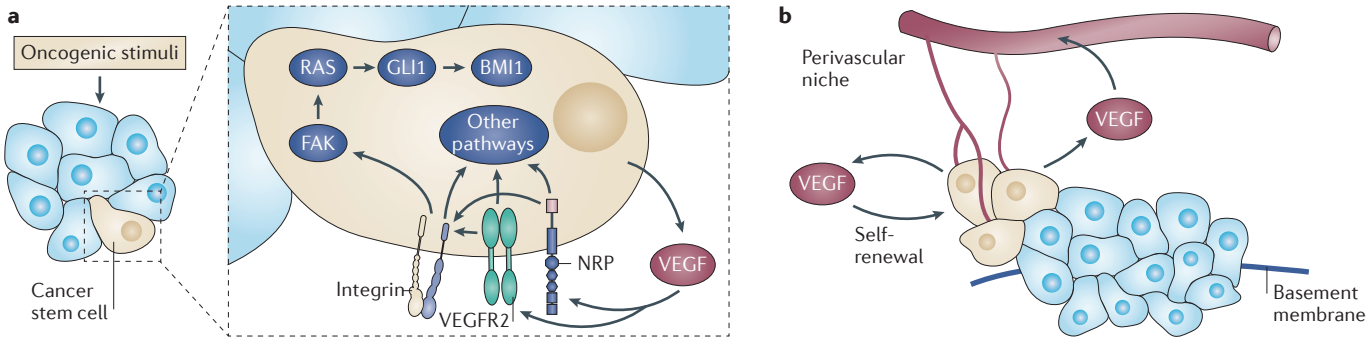

Figure 3 | **Role of autocrine VEGF signalling in the function of cancer stem cells and tumour formation.** **a** | The expression of vascular endothelial growth factor (VEGF) and VEGF receptors is induced concomitantly with oncogenic transformation; this facilitates the establishment of autocrine VEGF signalling. This signalling, which is mediated by the receptor tyrosine kinase VEGF receptor 2 (VEGFR2) and by neuropilins (NRPs), could be necessary for the function of cancer stem cells (beige cells) because it seems to maintain the size of the stem cell pool and to sustain self-renewal. The ability of autocrine VEGF signalling that is mediated by NRPs and integrins to regulate the expression of the Hedgehog target GLI family zinc finger 1 (GLI1) and the Polycomb group repressor BMI1 provides one mechanism to account for the contribution of autocrine VEGF signalling to the function of cancer stem cells, but other mechanisms probably exist. **b** | Cancer stem cells can be localized in a perivascular niche, which enables VEGF that is secreted by these cells to function in a paracrine manner to stimulate angiogenesis in nascent tumours. Autocrine VEGF signalling can also promote dedifferentiation and an epithelial–mesenchymal transition (EMT) phenotype that results in increased migration and invasion into the stroma. FAK, focal adhesion kinase.

which in turn activates GLI1. GLI1 then induces the expression of BMI1 (REF. 38) (FIG. 3), which is a Polycomb group transcriptional repressor that has been implicated in self-renewal and tumour initiation[98,99]. As mentioned above, GLI1 can stimulate the transcription of *NRP2* (REFS 38,52), which thereby creates a positive feedback loop that has the potential to sustain the self-renewal properties of cancer stem cells. Hedgehog signalling also has a crucial role in tumour cell–stromal cell interactions in this context, and this is shown by the finding that tumour-derived sonic hedgehog stimulates PLGF expression in stromal cells, which promotes the growth of medulloblastomas[80].

However, a fundamental issue is whether VEGF signalling alone can cause oncogenic transformation. Although there is a report that VEGFR1 can cause transformation[100], a more feasible hypothesis is that autocrine VEGF signalling is induced at the same time as other oncogenic events that drive tumour initiation, but that it can be an important (if not essential) component of the initiation process, as described above. In this context, a thought-provoking finding is that chronic inflammation causes upregulation of VEGFR2 in intestinal epithelial cells and that VEGFR2 signalling in these cells is required for tumour growth; this finding provides a causal link between inflammation and cancer that involves VEGF signalling[13]. However, this discussion of inflammation focuses on the role that autocrine VEGF signalling has in maintaining the function of cancer stem cells. Moreover, the data that are currently available clearly implicate autocrine VEGF signalling in sustaining self-renewal[38,40], which is consistent with the more general hypothesis that autocrine signalling is required to maintain a stem cell state[101]. Autocrine VEGF signalling is also closely associated with tumour dedifferentiation and with EMT[46], which are processes that may be involved in the genesis of cancer stem cells[101,102]. Moreover, autocrine VEGF signalling has been implicated in the metastatic cascade[36,80], and this is consistent with the recently identified role of cancer stem cells in tumour dissemination[102].

Although other growth factors may be involved in the autocrine signalling pathways that contribute to the function of cancer stem cells, VEGF is increasingly becoming recognized as a crucial factor. A potential explanation for this phenomenon is that the mechanisms that regulate VEGF expression — especially HIF-mediated transcription — are essential components of a stem cell phenotype[95,103]. The argument can be made that autocrine VEGF expression is a manifestation of HIF activation that is associated with the genesis of cancer stem cells and that is characteristic of poorly differentiated tumours[49,95]. For example, in high-grade prostate carcinoma, VEGF, HIF1α and NRP expression is higher in poorly differentiated tumour cells compared with more differentiated tumour cells[46].

Therapy

VEGF-targeted therapy — either alone or in combination with chemotherapy — is used for the treatment of many cancers[8]. Antibody-mediated inhibition of VEGF using bevacizumab is currently the predominant mode of VEGF-targeted therapy, although drugs that inhibit VEGF RTK activity (such as sunitinib and sorafenib) are also used[8]. The prevailing idea is that such therapy targets angiogenesis and other endothelial cell functions, and this aspect of VEGF-targeted therapy has been extensively studied and reviewed[8,104,105]. In this Review, we are interested in the possibility that targeting VEGF and VEGF receptors specifically on tumour cells could be effective in light of our increasing understanding of the importance of autocrine VEGF signalling in tumour initiation and in the biology of aggressive cancers. However, a

Polycomb group
Proteins that were first described in *Drosophila melanogaster* and that are required for normal development. They work in multiprotein complexes that are called Polycomb repressive complexes, which establish regions of chromatin in which gene expression is repressed.

potential caveat could be that although VEGF-targeted therapy (primarily bevacizumab) has reduced tumour burden and improved survival in some cancers, it has not been as successful as initially anticipated[106]. If we assume that bevacizumab has the potential to inhibit autocrine VEGF signalling in tumour cells, the modest effect of this drug that has been observed so far would diminish the importance and therapeutic potential of targeting VEGF signalling in tumour cells. However, a crucial observation is that bevacizumab does not inhibit the interaction of VEGF with NRPs[107]. Given the importance of NRPs to cancer stem cells and to VEGF signalling in tumour cells, which has been established in preclinical studies, this observation has widespread therapeutic implications and indicates that therapies that target NRPs or VEGF–NRP interactions could be very effective, especially when they are used in combination with antibodies against VEGF[108] (FIG. 4). Interestingly, clinical trials involving patients with advanced gastric and breast cancer assessed the efficacy of bevacizumab and concluded that high NRP1 expression is prognostic of a poor response to bevacizumab[109,110], reinforcing the importance of targeting NRP and the need for combination therapy.

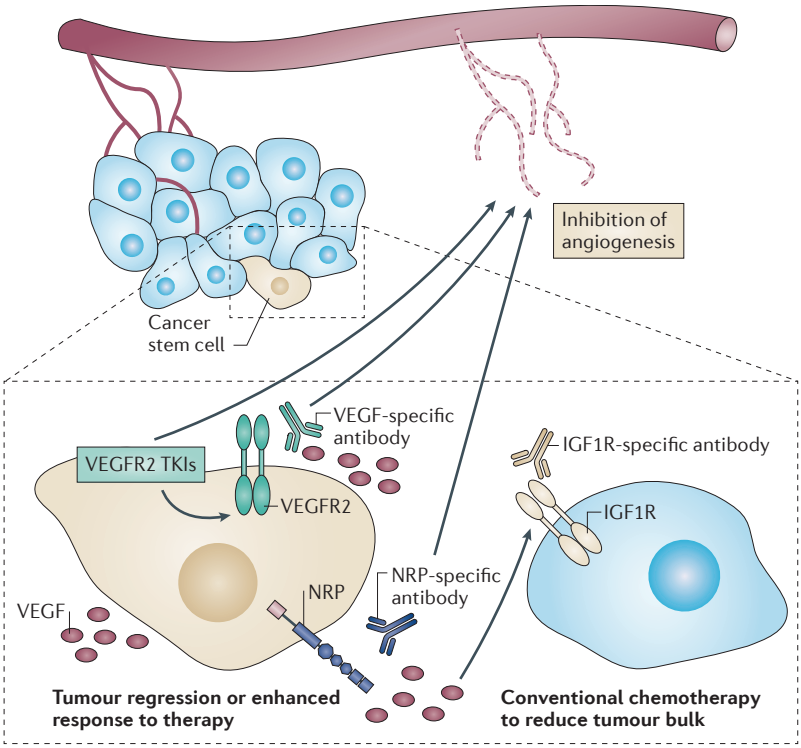

Figure 4 | **Therapeutic targeting of VEGF signalling in tumour cells.** The functional importance of vascular endothelial growth factors (VEGFs) and VEGF receptors — that is, neuropilins (NRPs) and VEGF receptor tyrosine kinases (RTKs) — that are expressed by tumour cells, in particular those that are expressed by cancer stem cells (beige cells), provides an important opportunity for the development of new therapeutic approaches, especially for highly aggressive tumours. These approaches have the potential to promote tumour regression and to improve the response to standard chemotherapy and radiation therapy. So far, strategies that inhibit VEGF signalling have primarily focused on targeting angiogenesis using either bevacizumab to inhibit VEGF or tyrosine kinase inhibitors (TKIs) that target VEGF RTKs such as VEGF receptor 2 (VEGFR2). NRPs are becoming recognized as crucial effectors of autocrine VEGF signalling in tumours, and more emphasis should be placed on targeting them therapeutically. Although some side effects were observed during the initial clinical use of a humanized NRP1 antibody, targeting NRPs is still a potentially effective strategy, and approaches need to be developed that minimize these side effects. It is also important to note that bevacizumab does not inhibit the binding of VEGF to NRPs, which highlights the importance of targeting NRPs directly and developing VEGF-specific reagents (such as placental growth factor (PLGF)-specific antibodies) that inhibit NRP binding. Targeting NRPs can result in compensatory signalling by other growth factor receptors, which indicates the potential importance of using a combination therapy. This possibility is shown by the compensatory insulin-like growth factor 1 receptor (IGF1R) signalling that occurs in prostate cancer, and it is likely that other mechanisms of compensation in response to VEGF pathway inhibition will be discovered for other tumour cells. These approaches may benefit from the use of conventional chemotherapy to reduce overall tumour burden.

Targeting NRPs and VEGF RTKs. Preclinical data suggests that targeting NRPs could potentially be used as a mode of cancer therapy. NRP expression is minimal in most adult tissues, which reduces the possibility that NRP-based therapies would perturb normal tissue function, and mouse studies using therapeutic NRP-specific antibodies have reported minimal side effects[108,111]. In addition, function-blocking NRP1- and NRP2-specific antibodies have been shown to inhibit tumour growth and to cause stasis in mice[38,80,108,112]. Similar results have been achieved using RNA interference (RNAi) to deplete NRP expression[113]. Other therapeutic approaches include the use of small peptides that prevent NRP oligomerization[114] or soluble forms of NRPs that function as decoy receptors[107]. Importantly, most of these studies concluded that targeting NRPs had a direct effect on tumour cells *in vivo*, and some studies showed that there was little effect on tumour angiogenesis[38].

Unfortunately, recent Phase I results using MNRP1685A, which is a humanized NRP1-specific monoclonal antibody, have raised concerns about targeting NRPs for therapy. MNRP1685A is cleared from the circulation more rapidly than other humanized antibodies, which suggests that it may have off-target effects[115]. Another concern is the preliminary report that patients who were dosed with MNRP1685A in combination with bevacizumab showed frequent but transient decreases in platelet levels and clinically significant proteinuria[116]. Given that NRP1 can affect the function of multiple growth factor receptors, these effects of MNRP1685A may not be surprising. However, these results should not discourage future work on the therapeutic targeting of NRPs, given the biological importance of NRPs in cancer. Strategies that target NRPs on tumour cells or cancer stem cells more specifically may decrease potential toxicity and off-target effects. The discovery that peptides with a carboxy-terminal arginine residue bind to NRP1 and NRP2 on the cell surface has been exploited as a novel approach to deliver cytotoxic peptides to tumour cells, and such tumour-penetrating peptides can be used to facilitate the delivery of co-administered drugs directly to tumour tissue[109,117]. Another novel NRP-targeting method that has important implications for therapy comes from our work that showed that the inhibition of NRP2 in prostate cancer cells induces the expression of the insulin-like growth factor 1 receptor (IGF1R) and triggers downstream signalling, which increases tumour proliferation[15]. However, NRP2 and IGF1R combination

therapy proved to be effective in reducing tumour burden (FIG. 4). This study also found that NRP2 is a valid biomarker for predicting the response to IGF1R therapy.

Some studies have investigated the effect of blocking VEGF RTKs or their activity in tumour cells[14,40,118,119]. Of note, antibody-mediated inhibition of VEGFR2 in the mouse model of skin cancer that is discussed above caused tumour regression by reducing the cancer stem cell pool size and by impairing cancer stem cell renewal, as well as by decreasing microvascular density[40]. In addition, the inhibition of VEGFR2 expression or activity blocked the VEGF–VEGFR2–NRP1 signalling axis. This inhibition also impeded the viability of glioma stem cells *in vitro* and increased the survival of mice that harboured glioma xenografts[14]. The interesting result of this study, which has been alluded to above, is that VEGFR2 signalling occurs intracellularly and is blocked by inhibitors of VEGFR2 tyrosine kinase activity but not by bevacizumab. In this context, it is thought-provoking that the inhibition of VEGFR2 expression in ovarian carcinoma cells has been shown to result in increased tumour growth *in vivo* and was associated with increased VEGF and NRP1 expression[119]. This finding confirms the importance of NRPs in tumour cells[119].

Targeting VEGF. Despite the concerns that relate to bevacizumab, the targeting of VEGF family members has the potential to be a very effective approach for inhibiting tumour cell function. This is supported by the report that bevacizumab treatment of patients with locally advanced breast cancer significantly increased tumour cell apoptosis[120]. In addition, antibody-mediated inhibition of PLGF in medulloblastomas had a direct antitumour effect *in vivo* and caused tumour regression[80], and it had minimal side effects. Similar results were achieved by blocking NRP1, but VEGFR1 inhibition had no effect. These findings substantiate the feasibility of using antibodies that are specific for other VEGF family members that block binding to NRPs. The potential of targeting VEGF is validated by the suppression of pancreatic carcinoma cell tumorigenesis by using a 'VEGF-trap' that sequesters VEGF[121].

The above findings are tempered by the report that anti-angiogenic therapy involving inhibition of either VEGF or VEGF RTKs increased tumour invasion and metastasis[122]. Of particular relevance, conditional deletion of *VEGFA* in pancreatic carcinoma cells, which should disrupt autocrine VEGF signalling, increased tumour invasiveness. By contrast, conditional deletion of *VEGFA* in established squamous carcinomas caused tumour regression[40]. An explanation for these seemingly contradictory findings is that *VEGFA* deletion inhibits slow-cycling cancer stem cells and selects for cells that proliferate at a higher rate and that may — at least in the short-term — be invasive. This hypothesis is supported by the above-mentioned finding that NRP2 inhibition in prostate cancer, which targets a putative stem cell population, increases IGF1R-mediated cell proliferation[15]. Although more work is needed to understand these fundamental issues, it is becoming evident that combined modes of therapy will be necessary to target VEGF signalling in tumour cells.

Conclusions and future perspectives

The salient theme of this Review is that VEGF signalling in tumour cells — especially autocrine signalling — can be an essential component of tumour initiation and it can be intimately associated with oncogenic transformation. More specifically, compelling data indicate that VEGF signalling promotes the function of cancer stem cells and sustains their self-renewal. These functions of VEGF are independent of its contribution to angiogenesis and, for this reason, constitute a paradigm shift in our understanding of the role of VEGF in cancer. Continued work to understand the relationship between autocrine VEGF signalling and the biology of cancer stem cells is warranted; in particular, because of the potential of using VEGF signalling as a therapeutic target.

VEGF signalling in tumour cells can involve VEGF RTKs, other RTKs, NRPs and integrins, but NRPs seem to be at the centre of signalling events that enable VEGF to affect tumour cell function, especially tumour initiation and the function of cancer stem cells. Much remains to be learnt about how NRPs function in this context, but it is evident that they have the ability to regulate the function and the trafficking of RTKs and integrins. An important issue in the future will be determining the extent to which NRP regulation of other receptors involves VEGF. Moreover, how this regulation occurs is only beginning to be understood, and the possibility that NRPs are components of macromolecular signalling complexes merits particular attention. NRPs may also have some semi-autonomous signalling potential that derives from their ability to interact with PDZ domain-containing proteins. Both NRP1 and NRP2 have been implicated in the function of cancer stem cells and are thought to have other functions in tumours, but the relative contributions of these two receptors is not yet known. Related issues are whether the NRP2 cytoplasmic domain variants have functional differences and the extent to which NRP glycosylation affects their ability to promote tumour initiation. It is somewhat surprising that the contribution of VEGF RTKs to VEGF signalling in tumour cells has not been consistent among studies — especially given the dominant role of VEGFR2 in driving angiogenesis — and it will be important to understand why their functional importance is diminished in some tumour cells.

The realization that autocrine VEGF signalling can be crucial for tumour initiation and for the characteristics of highly aggressive cancers provides a promising opportunity for the development of new therapeutic approaches. Such approaches are particularly intriguing because NRPs seem to be essential for this VEGF signalling and can be therapeutically targeted using currently available reagents. However, this excitement is tempered by the complexities that are associated with targeting VEGF and VEGF receptors, including potential toxicity, the possibility that cells resistant to such therapy can be highly aggressive and the possibility that compensatory signalling mechanisms may offset potential benefits. The development of more effective strategies will probably involve approaches that target tumour cells more specifically as well as the use of a combination of therapeutic reagents that overcome the resistance caused by targeting single molecules.

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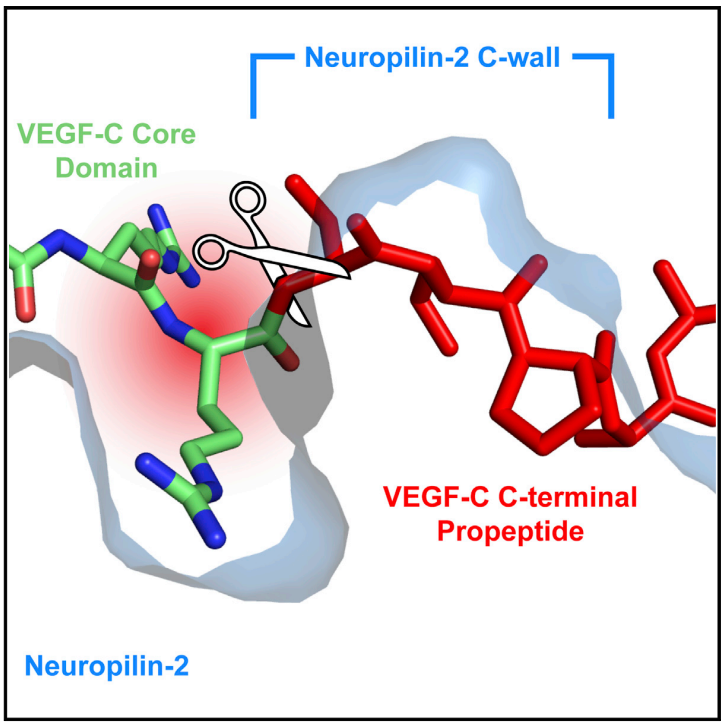
Competing interests statement
The authors declare no competing interests.

Article

Structure

Structural Basis for VEGF-C Binding to Neuropilin-2 and Sequestration by a Soluble Splice Form

Graphical Abstract



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In Brief

Vascular endothelial growth factor C (VEGF-C) is a potent lymphangiogenic cytokine that signals via the cell surface receptor Neuropilin-2 (Nrp2). Parker et al. demonstrate that VEGF-C binding to Nrp2 is regulated by C-terminal proteolytic maturation and identify a secreted splice form that functions as a selective inhibitor.

Highlights

- C-terminal processing of VEGF-C regulates direct binding to Nrp2
- Structure of the VEGF-C/Nrp2 complex demonstrates the basis for ligand binding
- Alternative Nrp2 splicing produces a soluble, dimeric receptor with novel structure
- Soluble Nrp2 functions as a potent antagonist of VEGF-C/Nrp2 binding

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Structure
Article

Structural Basis for VEGF-C Binding to Neuropilin-2 and Sequestration by a Soluble Splice Form

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SUMMARY

Vascular endothelial growth factor C (VEGF-C) is a potent lymphangiogenic cytokine that signals via the coordinated action of two cell surface receptors, Neuropilin-2 (Nrp2) and VEGFR-3. Diseases associated with both loss and gain of VEGF-C function, lymphedema and cancer, respectively, motivate studies of VEGF-C/Nrp2 binding and inhibition. Here, we demonstrate that VEGF-C binding to Nrp2 is regulated by C-terminal proteolytic maturation. The structure of the VEGF-C C terminus in complex with the ligand binding domains of Nrp2 demonstrates that a cryptic Nrp2 binding motif is released upon proteolysis, allowing specific engagement with the b1 domain of Nrp2. Based on the identified structural requirements for Nrp2 binding to VEGF-C, we hypothesized that the endogenous secreted splice form of Nrp2, s_9Nrp2, may function as a selective inhibitor of VEGF-C. We find that s_9Nrp2 forms a stable dimer that potently inhibits VEGF-C/Nrp2 binding and cellular signaling. These data provide critical insight into VEGF-C/Nrp2 binding and inhibition.

INTRODUCTION

The vascular endothelial growth factor (VEGF) family of cytokines are critical regulators of endothelial cell function. There are five VEGF family members: VEGF-A, -B, -C, -D, and placental growth factor (PlGF). Of these five, VEGF-C and VEGF-D selectively control lymphangiogenesis. While they show partially overlapping biological activity and physical properties, VEGF-C is essential for viability, whereas VEGF-D is not (Baldwin et al., 2005; Karkkainen et al., 2004). Endothelial cells of homozygous VEGF-C knockout mice do not sprout to form lymphatic vessels, which results in an alymphatic embryo and embryonic lethality (Karkkainen et al., 2004). Overexpression of VEGF-C results in selective induction of lymphatic but not vascular endothelial cell proliferation and lymphatic vessel enlargement (Jeltsch et al., 1997). In addition to its critical physiological role, VEGF-C signaling is also important for pathological lymphangiogenesis, which is associated with both aberrant loss of function in lymphedema (Saaristo et al., 2002) and gain of function in tumorigenesis and metastasis (Caunt et al., 2008; Ellis, 2006; Stacker et al., 2002).

VEGF-C signals via the coordinated activity of two families of endothelial cell surface receptors, the VEGF receptor (VEGFR) family of receptor tyrosine kinases (RTK) (reviewed in Stuttfeld and Ballmer-Hofer, 2009) and the Neuropilin (Nrp) family of coreceptors (reviewed in Parker et al., 2012a). VEGF-C function is specifically mediated through VEGFR-2/3 (Joukov et al., 1996; Kukk et al., 1996; Lymboussaki et al., 1999) and Nrp2 (Karkkainen et al., 2001; Xu et al., 2010), with VEGF-C capable of simultaneously engaging both families of receptors (Favier et al., 2006). VEGFR-2/3 have dual functionality in both angiogenesis and lymphangiogenesis (reviewed in Lohela et al., 2009). In contrast, Nrp2 knockout mice display normal angiogenesis but abnormal lymphatic vessel development (Yuan et al., 2002), similar to the tissue-specific function observed in the VEGF-C knockout (Karkkainen et al., 2004). Intriguingly, it has also been demonstrated that Nrp2 can function in VEGF-C signaling independent of its role as a coreceptor for VEGFR (Caunt et al., 2008).

Each member of the VEGF family of ligands is produced in multiple forms by either alternative splicing (e.g., VEGF-A, -B, and PlGF) or proteolytic processing (e.g., VEGF-C and -D) (Holmes and Zachary, 2005). In all cases, an invariant core cystine-knot domain, which specifically interacts with VEGFR, is combined with a variable C-terminal domain. VEGF-C is synthesized as a proprotein with N- and C-terminal domains flanking the central core cystine-knot domain. Prior to secretion, the C-terminal propeptide is cleaved followed by extracellular cleavage of the N terminus (Joukov et al., 1997). These processing events critically alter both the physiological and pathological bioactivity of VEGF-C (Siegfried et al., 2003). The mature dual-processed VEGF-C shows dramatically enhanced stimulatory activity in situ (McColl et al., 2003) and loss of C-terminal processing ablates function in vivo (Khatib et al., 2010). However, the physical basis for the enhanced activity of the mature form of VEGF-C remains unclear and has been connected to different properties, including differential receptor binding and interactions with heparin/extracellular matrix (ECM) (Harris et al., 2013; Joukov et al., 1997; Karpanen et al., 2006). The role of VEGF-C proteolytic maturation in regulating Nrp2 binding is unknown.

The structural basis for VEGF-C binding to VEGFR-2/3 has recently been elucidated and was shown to involve the invariant cystine-knot domain of VEGF-C binding to the N-terminal

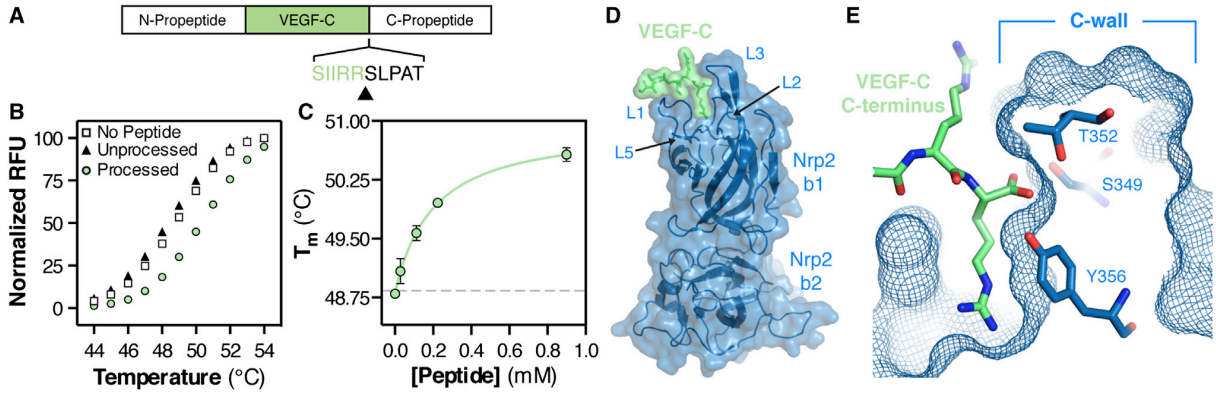

Figure 1. Crystal Structure of the VEGF-C/Nrp2 Complex Reveals the Basis for Proteolytic-Dependent Binding

(A) Organization of the VEGF-C proprotein and site of C-terminal processing (black arrow).

(B) Peptides corresponding to processed (green circle) and unprocessed (black triangle) VEGF-C were assayed for the ability to bind Nrp2-b1b2 as measured by DSF thermal shift assay. Peptides were added to Nrp2-b1b2 to a final concentration of 0.5 mM and melting was monitored between 20°C and 90°C. All samples were measured in triplicate, and a representative melting curve is shown for each. RFU, relative fluorescence units.

(C) Processed VEGF-C dose-dependently enhances the Nrp2-b1b2 T_m. Error bars indicate the SD of the three measurements.

(D) Structure of Nrp2-b1b2 (blue) in complex with the C terminus of VEGF-C (green).

(E) Cross-section of the Nrp2 binding pocket demonstrates that the free carboxy terminus of VEGF-C is buried against the Nrp2 C-wall, which is formed by the third coagulation factor loop.

domains of VEGFR-2 and VEGFR-3 (Leppanen et al., 2010, 2013). However, the structural basis for VEGF-C binding to Nrp2 remains to be determined. Alternative splicing and proteolysis modify the C-terminal variable region of VEGF and regulate Nrp binding (Makinen et al., 1999; Parker et al., 2012c; Soker et al., 1998). It has been demonstrated that Nrp1 binds the C-terminal basic domain of the Semaphorin-3 (Sema3) and VEGF family of ligands (He and Tessier-Lavigne, 1997; Soker et al., 1996), utilizing a binding pocket for ligands that contain a C-terminal arginine (Parker et al., 2010, 2012c; Vander Kooi et al., 2007; von Wronski et al., 2006). Importantly, the Sema3 family of ligands undergo furin-dependent proteolytic maturation within their C-terminal domain, a process that liberates an extended basic sequence and directly regulates bioactivity and Nrp binding (Adams et al., 1997; Parker et al., 2010, 2013).

Nrp2-dependent VEGF-C signaling is important in a variety of tumors and overexpression of these factors is correlated with advanced-stage disease and poor prognosis (Ellis, 2006; Stacker et al., 2002). Thus, specific Nrp2/VEGF-C inhibitors are of clinical interest. Soluble receptor fragments are common endogenous inhibitors (Albuquerque et al., 2009; Ambati et al., 2006; Kendall and Thomas, 1993; Rose-John and Heinrich, 1994). A soluble Nrp1 isoform was first identified as an endogenous inhibitor of prostate cancer in vivo (Gagnon et al., 2000). Soluble extracellular domain fragments can also be engineered for use clinically, including VEGF-trap (Aflibercept), a chimeric VEGFR-1/2-Fc fusion, which is an inhibitor of VEGF-A (Holash et al., 2002). A soluble splice form of Nrp2, s_9Nrp2, has been identified at the transcript level (Rossignol et al., 2000). s_9Nrp2 is produced by intron inclusion, which contains an in-frame stop codon. This stop codon is located prior to the transmembrane domain and is thus predicted to produce a secreted form of Nrp2. Interestingly, the insertion occurs in the middle of the second coagulation factor domain (b2), rather than in an interdomain region. The two Nrp2 coagulation factor domains (b1b2) form an integral unit (Appleton et al., 2007), and thus, the nature of the production and function of s_9Nrp2 is unclear. Further, domains b1b2 of Nrp2 have been demonstrated to bind VEGF-C (Karpanen et al., 2006), bringing into question whether this soluble splice form contains the structural requirements necessary to bind and sequester its ligands.

Here, we demonstrate that removal of the VEGF-C C-terminal propeptide directly regulates binding to Nrp2. The structure of the mature VEGF-C C terminus in complex with Nrp2 demonstrates that a cryptic Nrp2-binding motif is liberated upon C-terminal processing. This offers the first structural insight into the physical basis for VEGF-C binding to Nrp2, showing that the proteolytically liberated C-terminal arginine of VEGF-C directly binds the Nrp2 b1 domain. Mutagenesis of both VEGF-C and Nrp2 confirms the critical nature of the VEGF-C C-terminal sequence in Nrp2-b1 binding. Understanding the physical interactions underlying VEGF-C/Nrp2 binding led us to consider mechanisms for VEGF-C inhibition. The secreted Nrp2 splice form, s_9Nrp2, contains an intact Nrp2 b1 domain but a subsequent stop codon, and we assessed its function as a pathway-specific inhibitor. Strikingly, this soluble receptor forms a disulfide-linked dimer with two tightly integrated b1 domains and functions as a potent inhibitor of VEGF-C binding to Nrp2.

RESULTS

Structural Basis for Proteolytic-Dependent VEGF-C Binding to Nrp2

VEGF-C is synthesized as a proprotein with N- and C-terminal propeptides. Removal of the VEGF-C C-terminal propeptide critically regulates its bioactivity. C-terminal processing of VEGF-C liberates a polypeptide stretch rich in basic amino acids that terminates with a diarginine sequence (Figure 1A), a structural motif

Table 1. Data Collection and Refinement Statistics

Construct	Nrp2-VEGF-C	Nrp2-T319R	s_9Nrp2^B
Data Collection			
Beamline	APS 22-ID	APS 22-BM	APS 22-ID
Wavelength	1.0000	1.0000	1.0000
Space group	$P2_1$	$P2_12_12_1$	$P2_12_12$
Cell dimensions (Å)	41.05, 120.81, 69.84	34.90, 70.76, 122.97	69.36, 91.39, 67.33
Cell dimensions (°)	90.0, 103.29, 90.0	90.0, 90.0, 90.0	90.0, 90.0, 90.0
Unique reflections	44,081	12,223	16,303
Completeness (%)	90.6 (82.0)	96.4 (83.2)	94.1 (79.8)
Resolution (Å)	1.95 (2.02–1.95)	2.40 (2.49–2.40)	2.40 (2.49–2.40)
R_{merge} (%)	9.9 (46.6)	8.0 (29.2)	9.9 (32.7)
Redundancy	5.1 (4.2)	6.8 (5.9)	4.4 (4.1)
$I/\sigma(I)$	13.1 (3.0)	29.4 (5.1)	12.3 (3.2)
Refinement			
Resolution limits (Å)	20.00 (1.95)	20.00 (2.40)	20.00 (2.40)
No. reflections/no. to compute R_{free}	41,511/2,140	11,490/586	15,439/821
$R(R_{free})$	21.0 (24.1)	20.1 (25.5)	21.0 (26.4)
No. protein residues	632	313	361
No. solvent/ion molecules	333	123	107
Root-mean-square deviation bond (Å)	0.006	0.008	0.006
Root-mean-square deviation angle (°)	1.11	1.19	1.04
Protein Geometry			
Ramachandran outlier/favored (%)	0/96.7	0/96.1	0/96.7
Residues with bad bonds/angles	0/0	0/0	0/0
Rotamer outliers	0	0	0

conserved across the VEGF and Sema3 family of ligands and known to be important for Nrp1 binding. Thus, we hypothesized that processing of VEGF-C may directly regulate a physical interaction with Nrp2. To test this hypothesis, we produced peptides corresponding to the unprocessed (215-RQVHSIIRRSLPA-227) and processed (215-RQVHSIIRR-223) VEGF-C C terminus and measured the ability of each peptide to bind Nrp2 domains b1b2 using a differential scanning fluorimetry (DSF) thermal shift assay (Figure 1B). Processed VEGF-C significantly stabilized Nrp2-b1b2 (T_m 48.8°C ± 0.06°C to 50.3°C ± 0.05°C), while unprocessed VEGF-C showed no effect (T_m 48.4°C ± 0.04°C). Further, the processed VEGF-C peptide showed dose-dependent saturable binding to Nrp2-b1b2 with an apparent dissociation constant K_D = 199 μM ± 71 μM (Figure 1C). These data demonstrate that C-terminal proteolytic maturation directly regulates VEGF-C binding to Nrp2.

To define the physical basis for proteolytic-dependent binding of VEGF-C to Nrp2, we determined the crystal structure of the processed VEGF-C C terminus in complex with Nrp2 domains b1b2. The C-terminal five amino acids of mature VEGF-C (219-SIIRR-223), which are strictly conserved across species and also with VEGF-D, were fused to the C terminus of human Nrp2 domains b1b2 (residues 276–595). The fusion protein was expressed in *Escherichia coli*, purified, and crystallized. The structure was solved by molecular replacement and was refined to a resolution of 1.9 Å (Figure 1D; Table 1). There were two molecules in the asymmetric unit oriented in an antiparallel fashion (Figure S1A). Both molecules demonstrated specific binding of the VEGF-C encoded residues via an intermolecular interaction with a symmetry-related molecule.

Analysis of the structure reveals that VEGF-C (green) engages a binding pocket formed by the Nrp2-b1 (blue) coagulation factor loops (Figures 1D, S1B, and S1C). Indeed, this interloop cleft uniquely accommodates the C-terminal residue of processed VEGF-C (Figure 1E). The free carboxy terminus of VEGF-C is integrated into the binding pocket through interactions with residues from the third coagulation factor loop (L3) of Nrp2-b1, which form a wall at one side of the binding pocket (C-wall). Specifically, an extensive hydrogen bond network forms between the VEGF-C-free C-terminal carboxylate and the side chains of the C-wall residues S349, T352, and Y356 (Figure 1E). Importantly, the position of the C-wall would preclude binding of the unprocessed protein, providing a physical mechanism for the observed proteolytic-dependent binding of VEGF-C to Nrp2-b1b2.

Characterization of the VEGF-C/Nrp2 Interaction

Clear electron density for the VEGF-C-encoded region was observed, permitting modeling of both the VEGF-C polypeptide and interfacing solvent that bridge the two molecules (Figure 2A). Analysis of the VEGF-C/Nrp2 interface reveals direct interactions between VEGF-C and residues within the L1, L5, and L3 loops of Nrp2-b1 (Figure 2B), the regions that show the largest conformational changes when comparing the bound structure with the

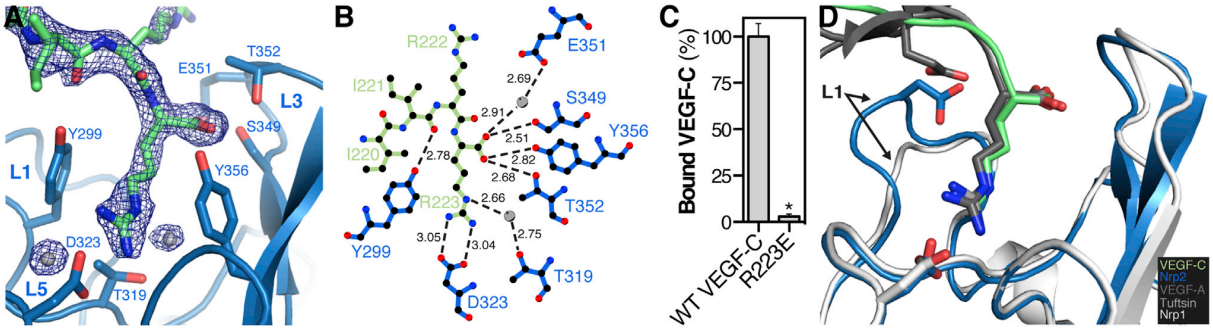

Figure 2. Mechanism of VEGF-C Binding to Nrp2

(A) Zoom of the intermolecular interface between Nrp2 (blue) and VEGF-C (green) with the $2F_o - F_c$ electron density map for VEGF-C contoured at 1.0σ. Interfacing water is shown as gray spheres.

(B) Ligplot+ generated representation of the interaction between VEGF-C (green) and Nrp2 (blue). Bond distances (Å) are labeled in black, and water is shown as gray spheres.

(C) Nrp2 binding was compared between VEGF-C and VEGF-C R223E. Binding was measured in triplicate and is reported as mean ± SD (*$p < 0.05$). WT, wild-type.

(D) Superimposition of the VEGF-A HBD/Nrp1 complex (PDB 4DEQ) and the tuftsin/Nrp1 complex (PDB 2ORZ) onto the structure of the VEGF-C/Nrp2 complex demonstrates the shared and unique modes of engagement within this ligand/receptor family.

previously reported apo structure (Figure S2) (Appleton et al., 2007). In addition to the hydrogen bond network formed between the VEGF-C free carboxy terminus and the Nrp2 L3 loop, the side chain of the VEGF-C C-terminal arginine, R223, forms extensive interactions with the Nrp2 binding pocket. The guanidinium of VEGF-C R223 forms a salt bridge with the Nrp2-b1 L5 loop residue D323. In addition, the aliphatic portion of the R223 side chain displays extensive van der Waals interactions with two tyrosine residues of Nrp2-b1 that demarcate the sides of the binding pocket, Y299 (L1 loop) and Y356 (L3 loop). In addition to interactions mediated by VEGF-C R223, there is a hydrogen bond between the backbone carbonyl of I221 and the aromatic hydroxyl of Nrp2 Y299.

While protein-protein binding is primarily mediated by direct interactions between polypeptide chains, interfacing solvent also plays a critical role in stabilizing protein-protein complexes (Janin, 1999; Karplus and Faerman, 1994). Three water molecules, two of which bridge the interaction between VEGF-C and Nrp2, are observed in the binding site. One solvent molecule facilitates a water-mediated hydrogen bond between the side chain hydroxyl of Nrp2 T319, located at the base of the binding pocket, and the side chain guanidinium of VEGF-C R223 (Figure 2B). Likewise, a second solvent molecule bridges the side chain carboxylate of Nrp2 E351 and the free carboxylate of VEGF-C. These solvent-mediated interactions appear to further stabilize the position of the VEGF-C C terminus within the Nrp2-b1 binding pocket.

To confirm the critical role of the VEGF-C C terminus, we mutated the C-terminal arginine of VEGF-C to glutamate (R223E) and compared the ability of alkaline phosphatase (AP)-tagged VEGF-C and VEGF-C R223E to bind Nrp2-b1b2 affinity plates (Figure 2C). Robust binding was observed between AP-VEGF-C and Nrp2-b1b2, but R223E binding was reduced by >95%. These data demonstrate that the C-terminal arginine of mature VEGF-C is necessary for high-affinity Nrp2-b1b2 binding and confirm the importance of C-terminal propeptide processing within VEGF-C to produce a C-terminal arginine that allows avid engagement of Nrp2.

The interaction observed between Nrp2-b1 and VEGF-C buries 374 Å^2 surface area of the VEGF-C C terminus. This is comparable with that observed for the exon 8 encoded residues of VEGF-A (338 Å^2 buried surface area) (Figure 2D, dark gray) (Parker et al., 2012c) and tuftsin (328 Å^2 buried surface area) (Figure 2D, light gray) (Vander Kooi et al., 2007) which complex with an equivalent binding site on Nrp1-b1. Importantly, these ligands, like VEGF-C, also contain a C-terminal arginine. All three ligands traverse the L1 loop, an orientation that is maintained by the engagement of the carboxy terminus by the C-wall. Collectively, the shared use of a C-terminal arginine in VEGF-A and VEGF-C explains their ability to bind both Nrp receptors (Karpanen et al., 2006; Parker et al., 2012b), while electrostatic repulsion by the L1 loop and adjacent regions account for receptor selectivity (Figure 2D) (Parker et al., 2012b, 2012c).

Occluding the Nrp2 Interloop Cleft Abolishes Binding

The structure of VEGF-C in complex with Nrp2 reveals a critical role for the Nrp2-b1 interloop cleft, which forms the VEGF-C binding pocket. To confirm that the Nrp2 binding pocket is responsible for VEGF-C binding, we carried out site-directed mutagenesis of Nrp2-b1b2 to generate a construct with an occluded binding pocket. Specifically, T319, a residue at the base of the Nrp2-b1 interloop cleft, was mutated to arginine (Nrp2-T319R). We determined the crystal structure of Nrp2-T319R to a resolution of 2.4 Å (Figure 3A; Table 1). The R319 side chain showed clear electron density extending into the interloop cleft between the two binding pocket tyrosines, Y299 and Y356 (Figure 3B). Superimposing the VEGF-C/Nrp2 complex onto Nrp2-T319R demonstrates that the binding site occupied by VEGF-C is occluded in the Nrp2 mutant (Figure 3C). The Nrp2-T319R mutant was then used to analyze the contribution of the interloop cleft to VEGF-C binding. We compared the binding of VEGF-C with Nrp2-b1b2 and Nrp2-T319R (Figure 3D).

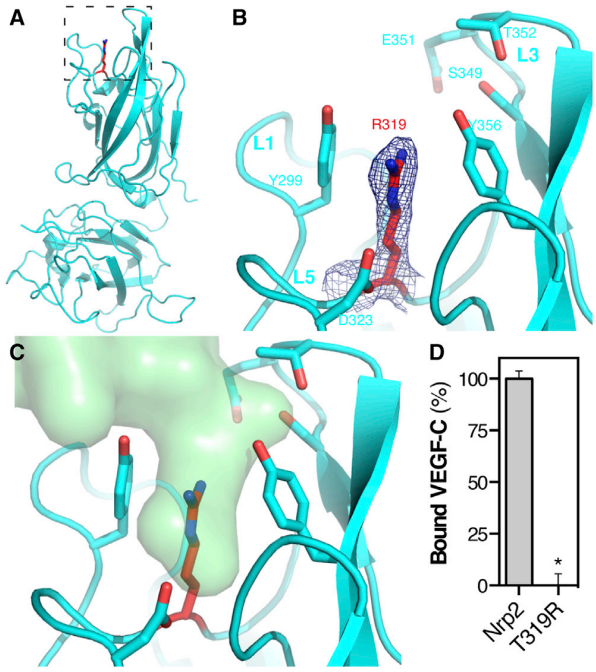

Figure 3. Crystal Structure and VEGF-C Binding Properties of Nrp2-T319R

(A) Structure of Nrp2-T319R with the stick representation for T319R shown in red.

(B) Zoom of the Nrp2-T319R binding pocket. The blue mesh illustrates the $2F_o - F_c$ electron density map for R319 contoured at 1.0σ.

(C) Superimposition of VEGF-C (green) onto the structure of Nrp2-T319R demonstrates that the binding pocket normally occupied by VEGF-C is blocked in the mutant.

(D) VEGF-C binding was compared between Nrp2-b1b2 and Nrp2-T319R. Binding was measured in triplicate and is reported as mean ± SD (*$p < 0.05$).

While robust binding was observed between AP-VEGF-C and Nrp2-b1b2, binding to Nrp2-T319R was completely abolished. These data confirm that the interloop cleft, formed by the Nrp2-b1 coagulation factor loops, forms a structure that uniquely accommodates the C terminus of VEGF-C to mediate binding of the C-terminally processed ligand.

A Dimeric Soluble Nrp2 Splice Form

Based on the specific binding of VEGF-C to the Nrp2 b1 domain, we hypothesized that the previously identified splice form of Nrp2, s_9Nrp2, could function as a selective inhibitor of VEGF-C. s_9Nrp2 is an alternative Nrp2 splice form that arises from intron inclusion in the b2 domain (Figure 4A). An in-frame stop codon encoded within the intron is predicted to result in termination of translation prior to the transmembrane domain, and thus production of a secreted Nrp2 receptor that contains the first two CUB domains (a1 and a2) and the first coagulation factor domain (b1), but only a portion of the coding sequence for the second coagulation factor domain (b2). Given that the b1 domain of Nrp2 is solely responsible for VEGF-C binding, we hypothesized that s_9Nrp2 may be able to effectively sequester VEGF-C, thereby functioning as an inhibitor. However, it is unknown whether the s_9Nrp2 transcript produces a functional protein, since s_9Nrp2 retains residues coding only a portion of the b2 domain (114 of 159 residues). Indeed, s_9Nrp2 lacks the coding region for three of the eight core β strands that normally integrate to form the distorted jelly-roll fold that typifies the b1 and b2 domains of Nrp. In addition, it was unknown whether s_9Nrp2 could accommodate the loss of the canonical C-terminal capping cysteine of the b2 domain. To investigate the physical and functional activity of s_9Nrp2, we tested the ability of this isoform to be secreted from eukaryotic cells. We produced s_9Nrp2 and a construct containing solely the ligand binding coagulation factor domains, s_9Nrp2^B (Figure 4A), as a human growth hormone (Hgh)-fusion in Chinese hamster ovary (CHO) cells. Western blot analysis demonstrated that both constructs were efficiently produced and secreted (Figure 4B). We next produced s_9Nrp2^B protein in bacteria. Analysis of s_9Nrp2^B by reducing SDS-PAGE revealed that purified s_9Nrp2^B, while running with a larger apparent molecular weight (MW) than Nrp2-b1 alone, was smaller than expected from its primary sequence (Figure 4C, observed MW = 22 kDa, expected MW = 34 kDa). Mass spectrometry confirmed that s_9Nrp2^B is an essentially homogeneous single species with MW = 22,775 Da ± 20 Da. These data, together with the observed intact N-terminal His-tag, indicate that s_9Nrp2^B is cleaved C-terminal to E457 (predicted MW = 22,792 Da). Thus, the proteolyzed s_9Nrp2^B contains only a single cysteine residue from the b2 domain (C434), which normally forms an intradomain disulfide. Surprisingly, under nonreducing conditions, s_9Nrp2^B ran with an apparent MW = 38 kDa, indicating the formation of a disulfide-linked intermolecular dimer via the free b2 domain cysteine (Figure 4C). Predominantly disulfide-linked dimeric protein is also observed in s_9Nrp2^B protein purified from CHO-cell conditioned media (Figure S3A). The difference in oligomeric state was evident from size exclusion chromatography (SEC) (Figure 4D). Nrp2-b1 eluted off SEC with an apparent MW = 16 kDa (gray line), while s_9Nrp2^B had an apparent MW = 38 kDa (black line), consistent with the SDS-PAGE analysis.

s_9Nrp2^B Is a Uniquely Potent Inhibitor of VEGF-C/Nrp2 Binding

To understand the structural arrangement of the s_9Nrp2^B dimer, we determined the crystal structure of s_9Nrp2^B to a resolution of 2.4 Å (Figure 5A; Table 1). Continuous electron density was observed from F275 to S453, consistent with the C terminus defined using mass spectrometry. A single dimer was present in the asymmetric unit, with the base of each b1 domain apposed to the other, thus forming an extended antiparallel dimer. The orientation of the dimer is stabilized by both the intermolecular disulfide and, unexpectedly, a unique dimeric helical bundle formed by residues from the b1-b2 linker and b2 domain (Figure 5B). The residues that form this unique helix (residues 428–453) display dramatic structural reorganization relative to that observed in the intact b2-domain, where they form an extended sheet and loop motif (Figure 5C). The C-terminal helix runs approximately 20° off parallel from the base of the b1 domain, an angle that is maintained by a cluster of hydrophobic residues at the hinge region between the helix and domain b1. The helix both caps the b1 domain and mediates the intermolecular interaction interface with the other monomer of the s_9Nrp2^B dimer.

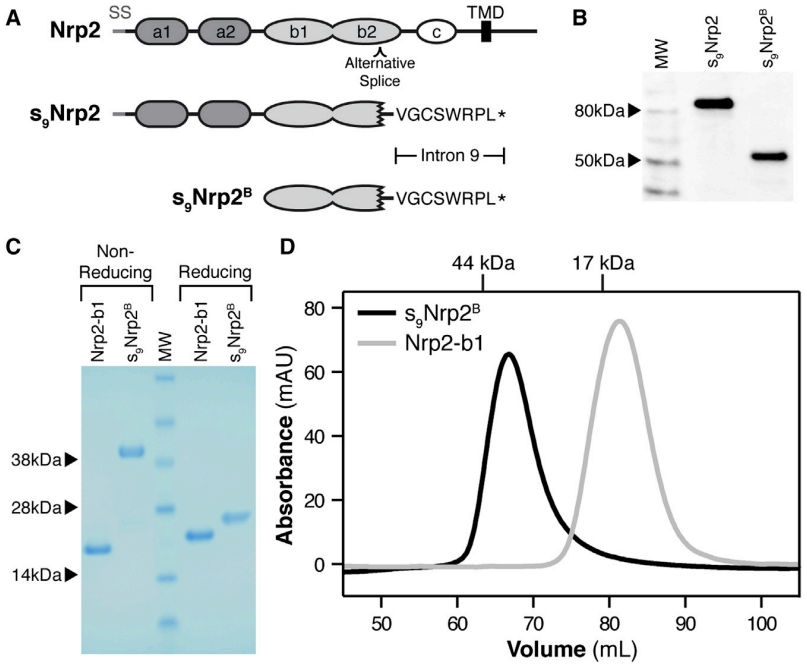

Figure 4. s_9Nrp2^B Forms a Disulfide-Linked Dimer

(A) Domain organization of Nrp2, s_9Nrp2, and the protein fragment used for our studies, s_9Nrp2^B. The intron 9-encoded sequence is indicated, which includes the in-frame stop codon (*).

(B) Western blot analysis of Hgh-tagged s_9Nrp2 and s_9Nrp2^B expressed in CHO cells.

(C) Nonreducing and reducing SDS-PAGE analysis of Nrp2-b1 and s_9Nrp2^B.

(D) The oligomeric state of s_9Nrp2^B (black line) was analyzed by size exclusion chromatography. Nrp2-b1 was run as a reference (gray line).

The intermolecular interface is composed of both helix-helix interactions, which are mostly hydrophobic in nature (Figure 5B), and helix-b1 interactions, which are mostly hydrophilic in nature. Truncation of the helix decreased the amount of dimeric species formed, demonstrating a role for the helix in the formation of a stable disulfide-linked dimer (Figure S3B).

The two binding pockets within the s_9Nrp2^B dimer are positioned 71 Å apart, suggesting that it could simultaneously engage both subunits of the VEGF-C dimer, which is 68 Å wide (Leppanen et al., 2010). Thus, we hypothesized that coengagement of both VEGF-C monomers by s_9Nrp2^B would allow the dimer to function as a uniquely potent inhibitor of VEGF-C/Nrp2 binding. To test this hypothesis, we compared the inhibitory potency of ATWLPPR, an optimized peptide inhibitor of Nrp that functions by competitive binding (Parker and Vander Kooi, 2014; Starzec et al., 2007), with Nrp2-b1 and s_9Nrp2^B, both of which function as soluble competitors through sequestration of VEGF-C (Figure 5D). ATWLPPR showed dose-dependent inhibition of VEGF-C binding to Nrp2 with an inhibitory concentration 50% (IC_{50}) = 10 μM (gray line), consistent with its modest reported potency. Next, we examined the ability of Nrp2-b1 to inhibit binding (blue line). Nrp2-b1 sequestered VEGF-C with improved potency compared with the peptide inhibitor, with an IC_{50} = 1.5 μM. As expected for a monomeric competitive inhibitor, the Hill slope was approximately –1 (ATWLPPR = –1.08 and Nrp2-b1 = –0.97). These data are consistent with independent engagement of each VEGF-C monomer by a single Nrp2-b1. Next, we measured the inhibitory potency of s_9Nrp2^B (orange line). Strikingly, s_9Nrp2^B potently sequestered VEGF-C with an IC_{50} = 250 nM, a significant improvement in potency from both the peptide inhibitor and Nrp2-b1. In addition, the Hill slope for s_9Nrp2^B was –1.5. Thus, the enhanced potency of s_9Nrp2^B is due to its ability to synergistically sequester the VEGF-C dimer through simultaneous and cooperative engagement of the two VEGF-C monomers.

VEGF-C signaling requires the coordinated action of Nrp2 and the RTK VEGFR3 (Xu et al., 2010). Nrp2 enhances signaling via VEGF-C through both a direct interaction with VEGF-C and via coupling with VEGFR3 (Favier et al., 2006). Thus, while s_9Nrp2^B could inhibit VEGF-C signaling by sequestering VEGF-C ligand from Nrp2, it is also possible that s_9Nrp2^B could interact with VEGFR3 and actually enhance VEGF-C binding and signaling. Therefore, we tested the effect of s_9Nrp2^B on VEGF-C binding to VEGFR3. While s_9Nrp2^B blocked VEGF-C binding to Nrp2, it showed no effect on VEGF-C binding to VEGFR3, indicating that the binding events are independent (Figure 5E).

We extended our studies to assess the efficacy of s_9Nrp2^B as an inhibitor of Nrp2 signaling in prostate cancer. Nrp2 and VEGF-C expression have both been reported to function in the survival and aggressiveness of prostate cancer (Goel et al., 2012; Muders et al., 2009). We assessed the ability of Nrp inhibition to reduce the formation of prostatospheres by C4-2 cells. Incubation with s_9Nrp2^B resulted in a significant decrease in prostatosphere formation (Figure 5F). Incubation with the specific Nrp inhibitor C-furSema (Goel et al., 2013; Parker et al., 2010) likewise significantly reduced prostatosphere formation, whereas the control C-Sema did not, demonstrating the specific role for Nrp in prostatosphere formation. These data demonstrate that s_9Nrp2^B can effectively sequester VEGF-C and raises the exciting possibility of using engineered Nrp ectodomains as inhibitors of pathological Nrp-dependent signaling (Figure 5G).

DISCUSSION

Structural characterization of the mechanism for VEGF-C binding to Nrp2 represents an important step for understanding the physiological and pathological activity of VEGF-C. These data also inform the rational design of specific VEGF-C/D antagonists, including s_9Nrp2^B, which potently inhibits VEGF-C/Nrp2 binding and represents a potential therapeutic avenue. Collectively, these results have important implications for interpreting both the aberrant loss and gain of function in the VEGF-C/Nrp2 signaling axis that critically underlies a number of disease states.

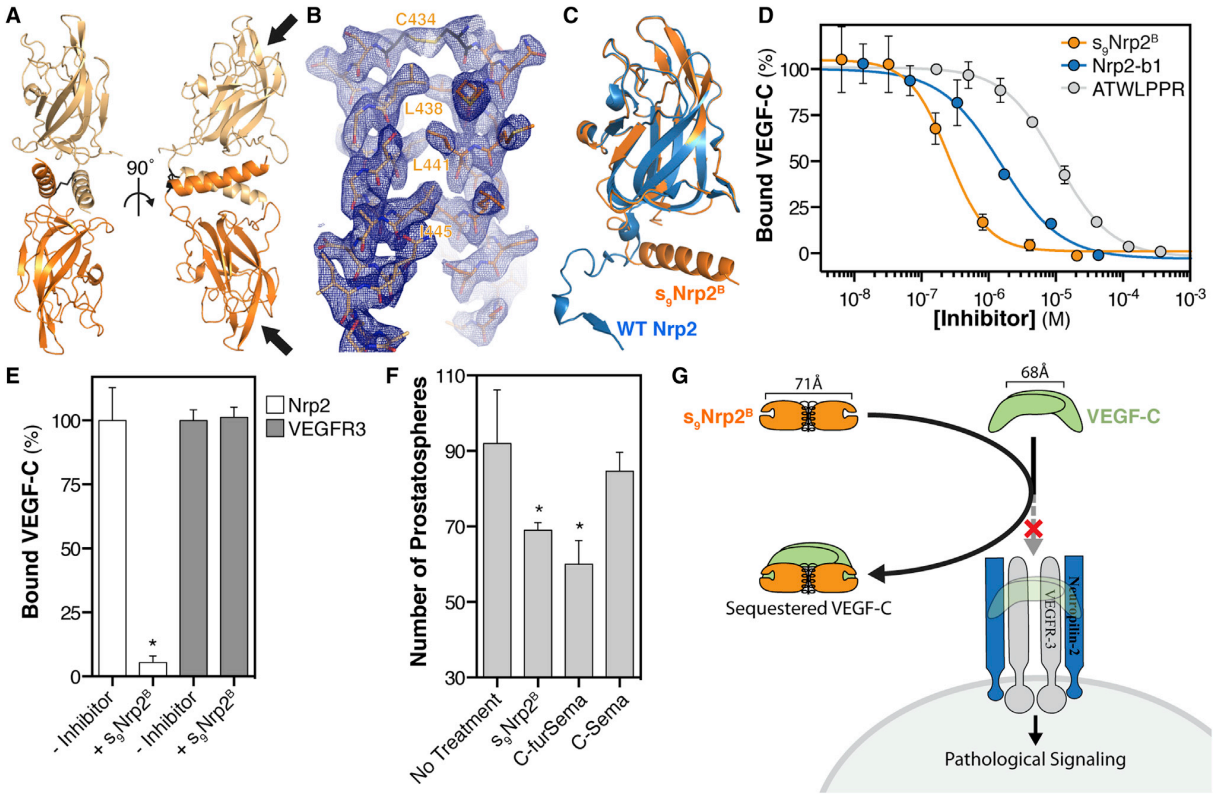

Figure 5. Crystal Structure and Inhibitory Properties of s_9Nrp2^B

(A) Crystal structure of the s_9Nrp2^B dimer (chain A, light orange; chain B, dark orange). The intermolecular disulfide is shown in black and the Nrp2-b1 binding pockets are labeled with arrows.

(B) Zoom of the dimeric helical bundle with the $2F_o - F_c$ electron density map contoured at 1σ.

(C) The residues of the Nrp2 b1-b2 linker and b2 domain show a dramatic structural reorganization from an extended loop in the b1b2 sequence (blue) to an extended helix in the s_9Nrp2^B dimer (orange).

(D) ATWLPPR (gray), Nrp2-b1 (blue), and s_9Nrp2^B (orange) were assayed for the ability to inhibit VEGF-C binding to Nrp2. ATWLPPR inhibited binding with an IC_{50} = 10 μM ($\log[IC_{50}] = -4.98 \pm 0.03$), Nrp2-b1 inhibited binding with an IC_{50} = 1.5 μM ($\log[IC_{50}] = -5.82 \pm 0.09$), and s_9Nrp2^B inhibited binding with an IC_{50} = 250 nM ($\log[IC_{50}] = -6.60 \pm 0.08$).

(E) s_9Nrp2^B was assayed for the ability to alter VEGF-C binding to VEGFR3. Addition of 4 μM s_9Nrp2^B fully inhibited VEGF-C/Nrp2 binding but showed no effect on VEGF-C/VEGFR3 binding.

(F) Inhibition of C4-2 cell prostatosphere formation was used to assess the biological activity of s_9Nrp2^B. Prostatosphere formation was compared in the absence and presence of s_9Nrp2^B, as well as with C-furSema (positive control) and C-Sema (negative control).

(G) Model illustrating the mechanism of action for s_9Nrp2^B. s_9Nrp2^B sequesters VEGF-C and prevents activation of the VEGFR3/Nrp2 signaling complex. All inhibition experiments were measured in triplicate and reported as mean ± SD (*$p < 0.05$).

With complementary biochemical and structural approaches, we show that VEGF-C C-terminal proteolysis is required for Nrp2 binding. The requirement for proteolytic processing is determined by the position of the Nrp2 C-wall, formed by the L3 coagulation factor loop residues, which specifically engages the VEGF-C free carboxy terminus, precluding binding of unprocessed protein. These results provide critical insight for interpreting the altered in vitro and in vivo functionality of alternative VEGF-C forms. While both N- and C-terminal processing regulate VEGF-C activity (Joukov et al., 1997; McColl et al., 2003), processing at these sites is not functionally equivalent. Indeed, loss of C-terminal processing is uniquely detrimental, fully ablating VEGF-C function in vivo (Khatib et al., 2010), which we demonstrate blocks Nrp2 binding.

The loss of VEGF-C binding to Nrp2-T319R, a mutant with an occluded binding pocket, demonstrates the use of a C-terminal arginine for ligand engagement. Indeed, the VEGF-C C-terminal arginine side chain and free carboxylate form extensive interactions with the Nrp2-b1 binding pocket. Interestingly, VEGF-C is not the only VEGF family member that, in the absence of post-translational modification, lacks a C-terminal arginine. Of the five VEGF family members, three contain Nrp binding domains that lack this structural motif (VEGF-C, VEGF-D, and VEGF-B_{186}). VEGF-D, a close structural and functional homolog of VEGF-C, is processed at an equivalent site in its C terminus to produce a C-terminal arginine (Stacker et al., 1999) and thus likely utilizes a similar binding mode to Nrp2. This observation provides additional functional insight, as loss of VEGF-D

C-terminal processing also ablates function in vivo (Harris et al., 2013). There are three VEGF-B isoforms, VEGF-B_{167}, VEGF-B_{127}, and VEGF-B_{186}, all of which differ in their C-terminal domain (Olofsson et al., 1996a, 1996b). Characterization of VEGF-B_{186} demonstrated that it exhibited proteolytic-dependent binding to Nrp1 and identified the site of proteolysis as R227 (Makinen et al., 1999). Thus, the mechanism of proteolytic-dependent VEGF-C binding to Nrp2 has broad explanatory power for understanding Nrp binding across the VEGF family.

Determining the structural basis for VEGF-C signaling via Nrp2 informs ongoing studies to describe the effect of signaling deficiency on human disease. Deficient VEGF-C signaling via Nrp2 has significant implications for both primary and secondary lymphedema. Mutations in both VEGFR-3 (Karkkainen et al., 2000) and VEGF-C (Gordon et al., 2013) have been demonstrated to underlie hereditary lymphedema and Nrp2 has been identified as an additional candidate gene (Ferrell et al., 2008; Karkkainen et al., 2001). In addition, both VEGF-C and Nrp2 have recently been identified as candidate genes for the development of secondary lymphedema following surgery in breast cancer (Miaskowski et al., 2013). The structural insights gleaned from the VEGF-C/Nrp2 complex also provide an important molecular basis for interpreting emerging exome sequencing data that has identified Nrp2 variants in close proximity to the ligand binding interface. Intriguingly, a stringent examination of exome sequencing data has reported both common and rare Nrp2 variants in human populations (Tennessen et al., 2012). Several of these variants are located in the coagulation factor loops of Nrp2-b1, the region to which VEGF-C binds. Specifically, there are two reported variants in the L5 loop (N321I and L322M), which are located proximal to the critical salt bridge formed by D323, and two in the L3 loop (Q353H and N354K). The structural data presented here provide a rationale for examining specific coagulation factor loop variants for loss of function on both a physical and functional level.

As opposed to aberrant VEGF-C loss of function in lymphedema, aberrant activation of VEGF-C signaling via Nrp2 is associated with cancer initiation, survival, and progression (Ellis, 2006; Stacker et al., 2002). The Nrp2/VEGF-C signaling axis contributes to tumorigenesis via multiple mechanisms. Mimicking its physiological function, VEGF-C signaling via Nrp2 stimulates lymphatic vessel recruitment to tumors and directly contributes to cancer metastasis (Caunt et al., 2008). Importantly, the role of VEGF-C and Nrp2 in tumorigenesis is not exclusively associated with aberrant lymphangiogenesis. Indeed, in situ studies have demonstrated that autocrine VEGF-C signaling in breast cancer cells stimulates cellular motility (Timoshenko et al., 2007). Further, recent reports indicate that cancer cell survival is enhanced through VEGF-C/Nrp2-dependent autophagy (Stanton et al., 2012) and that autocrine Nrp2 signaling maintains the population of cancer stem cells (Goel et al., 2013). VEGF-C also functions to protect prostate cancer cells from oxidative stress in an Nrp2-dependent fashion (Muders et al., 2009). Thus, selective inhibition of Nrp2 represents a promising, multipronged anticancer therapeutic strategy.

Secreted splice forms of angiogenic receptors have essential roles in vivo (Albuquerque et al., 2009; Ambati et al., 2006; Kendall and Thomas, 1993) and have been engineered to serve as therapeutic inhibitors that block aberrant pathway activation by ligand sequestration (Stewart, 2012). Here, we demonstrate that the alternative Nrp2 splice form, s_9Nrp2^B, potently sequesters VEGF-C and inhibits binding to Nrp2. The biological function and localized tissue-specific expression of s_9Nrp2 are of significant interest. Indeed, s_9Nrp2 may be analogous or complementary to sVEGFR-2, the secreted splice form of VEGFR-2 that functions as an endogenous lymphangiogenesis inhibitor (Albuquerque et al., 2009). VEGF-D also functions in lymphatic angiogenesis and has been shown to have partially overlapping biological function with VEGF-C and important pathological functions (Haiko et al., 2008; Harris et al., 2013; Karpanen et al., 2006). The conservation of Nrp2-interacting residues between VEGF-C and VEGF-D strongly suggests that s_9Nrp2^B will equivalently sequester both VEGF-C and VEGF-D. In contrast, the heparin-binding domain of VEGF-A contains specificity determinants that limit binding to Nrp2 (Parker et al., 2012b, 2012c). Thus, s_9Nrp2^B is likely to selectively sequester the lymphangiogenic-specific VEGF family members, VEGF-C and VEGF-D.

The practice of engineering inhibitor multimerization to increase potency is well established for soluble receptor fragments. Most commonly, soluble receptors are dimerized by expression as an Fc fusion protein (e.g., VEGF-trap). s_9Nrp2^B represents a unique mechanism for generation of a multimeric protein that maintains the benefits of avidity but does not require introduction of an exogenous polypeptide sequence. Additional optimization of s_9Nrp2^B potency, selectivity, and stability is an important future direction for the development of a therapeutically useful inhibitor.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification

Human Nrp2-b1b2 (residues 276–595), human Nrp2-b1 (residues 276–430), human Nrp2-T319R (residues 276–595 with T319R mutation), s_9Nrp2^B (residues 275–555: isoform O60462-6), s_9Nrp2^B-Δ-helix (residues 276–436), and the Nrp2-b1b2/VEGF-C fusion were expressed in *E. coli* as His-tag fusion proteins from pET28b (Merck). Proteins were purified via immobilized metal ion affinity chromatography (IMAC) and either heparin affinity or SEC. AP-VEGF-C (residues 108–223) wild-type and mutant and Hgh-tagged proteins were produced by transient transfection of CHO cells (Aricescu et al., 2006). The VEGFR-3 extracellular domain was produced via baculovirus-mediated expression (residues 21–776) and purified by IMAC and SEC.

Structure Determination

Purified Nrp2-b1b2-VEGF-C fusion, Nrp2-T319R, and s_9Nrp2^B were concentrated to 2.0 mg/ml, 2.1 mg/ml, and 3.5 mg/ml, respectively, and crystals grown by hanging-drop vapor-diffusion experiments. Fusion protein crystals were obtained in 2 weeks at room temperature (RT) in 0.1 M MES (pH 6.5), 0.5 M ammonium sulfate. Nrp2-T319R crystals were obtained in 5 days at RT in 0.1 M HEPES (pH 7), 18% (w/v) PEG 12000. s_9Nrp2^B crystals were obtained in 2 weeks at RT in 10% PEG 1000/10% PEG 8000. Crystals were passed through mother liquor supplemented with 10% glycerol and then flash frozen in liquid nitrogen. Diffraction data were collected at the SER-CAT 22-ID and 22-BM beamlines of the Advanced Photon Source, Argonne National Laboratories and processed using HKL2000 (Otwinowski and Minor, 1997). Structures were solved by molecular replacement using Nrp2-b1b2 (PDB 2QQJ) followed by iterative modeling building and refinement using COOT (Emsley et al., 2010) and Refmac5 (Murshudov, 1997) to generate a final refined model (Table 1).

DSF

Peptides corresponding to processed and unprocessed VEGF-C were produced with an N-terminal tryptophan to allow accurate quantitation by UV_{280} absorbance (LifeTein LLC). Peptides were resuspended and combined with

2 μM of Nrp2-b1b2 and 5x SYPRO Orange Protein Gel Stain (Life Technologies) in PBS. Nrp2-b1b2 melting was monitored on a CFX96 Real-Time PCR system (Bio-Rad) from 20°C to 90°C at a rate of 1°C/50 s with fluorescent readings taken every 1°C.

Binding and Inhibition Assays

Plate binding and soluble Nrp competition assays were performed by measuring the binding of AP-tagged VEGF-C to Nrp2-b1b2, Nrp2-T319R, or VEGFR3 affinity plates. For direct binding assays, ligand was directly added to Nrp2-affinity plates, incubated for 1 hr at RT, washed, and developed using *p*-nitrophenyl phosphate AP substrate. For competition experiments, ligand was premixed with inhibitor and then added to affinity plates as with binding.

Prostatosphere Assays

Prostatosphere cultures used C4-2 prostate cancer cells (UroCor) (Cao et al., 2011). 5,000 cells/well were cultured in suspension in serum-free DMEM-F12 (Life Technologies), supplemented with B27 (1:50, Life Technologies), 20 ng/ml epidermal growth factor (Peprotech), and 4 μg/ml insulin (Sigma-Aldrich) in 6-well ultralow attachment plates (Corning). s_9Nrp2^B, C-furSema, or C-Sema inhibitors were added at a concentration of 5.0 μM while plating the cells. The prostatospheres were cultured for 6 days, and 1 ml of culture medium was added every other day. Spheres larger than 100 μm were counted.

ACCESSION NUMBERS

Coordinates and structure factors have been deposited in the PDB, www.pdb.org, with accession codes 4QDQ, 4QDR, 4QDS for the complex, T319R, and dimer structures, respectively.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at http://dx.doi.org/10.1016/j.str.2015.01.018.

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